2014

Characterizing Arabidopsis thaliana mutant lines that affect leaf shape and vein pattern

Liu, Chen

Lethbridge, Alta. : University of Lethbridge, Dept. of Biological Sciences

http://hdl.handle.net/10133/3518

Downloaded from University of Lethbridge Research Repository, OPUS
CHARACTERIZING ARABIDOPSIS THALIANA MUTANT LINES THAT AFFECT LEAF SHAPE AND VEIN PATTERN

CHEN LIU
B.Sc., Qilu University of Technology, China, 2010

A Thesis
Submitted to the School of Graduate Studies of the University of Lethbridge in Partial Fulfilment of the Requirements for the Degree

MASTER OF SCIENCE

Biological Sciences
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

© Chen Liu, 2014
CHARACTERIZING ARABIDOPSIS THALIANA MUTANT LINES THAT AFFECT LEAF SHAPE AND VEIN PATTERN

CHEN LIU

Date of Defence: August 11, 2014

Dr. Elizabeth Schultz
Supervisor
Associate Professor       Ph.D.

Dr. Alicja Ziemienowicz
Thesis Examination Committee Member
Associate Professor       Ph.D.

Dr. Larry Flanagan
Thesis Examination Committee Member
Professor                  Ph.D.

Dr. Tony Russell
Thesis Examination Committee Member
Assistant Professor       Ph.D.

Dr. Theresa Burg
Chair, Thesis Examination Committee
Associate Professor       Ph.D.
I dedicate this thesis to my supervisor Dr. Elizabeth Schultz and my family for their consistent support and encouragement
ABSTRACT

An optimum leaf shape and vein pattern is necessary for vascular plant success. The isolation of leaf morphological mutants of Arabidopsis thaliana has been used for leaf development studies. Auxin is a signal molecular that regulates plant development and growth processes. I have analyzed four mutations in detail, and I suggest that they all have defects in auxin regulated plant development processes. Mutant 115-11-21-4 is an allele of FKDI with frequent distal free-ending veins. Mutant 31-83-4-3 likely has increased auxin flux through secondary veins and increased downward auxin transport through the shoot, possibly due to increased PIN1 expression and/or altered PIN1 localization. Mutant 32-2-4 likely has increased auxin activity at marginal auxin convergence points resulting in the formation of increased leaf serrations. Mutant 33-7-5(4) may have a global reduction in auxin biosynthesis or response, thus the whole plant is weak and pale, with slower shoot and root growth rate and gravitropic response.
ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor, Dr. Elizabeth Schultz, Associate Professor of Biology, for her consistent guidance, support and encouragement throughout my program. In these three years, I learned a lot from her distinguished expertise and I am honored to be a part of her research team. I wish to acknowledge all my supervisory committee members, Dr. Alicja Ziemienowicz, Associate Professor of Biology, Dr. Larry Flanagan, Professor of Biology, and Dr. Tony Russell, Assistant Professor of Biology, for their critical suggestions, valuable comments and insightful discussions on my academic course study and research program.

I appreciate Dr. Dongping Li, Post Doctorate of Biology, Dr. Bo Wang, Post Doctorate of Biology and Dr. Youli Yao, Post Doctorate of Biology for helping me with experiment trouble shooting. I would like to thank the members of Schultz’s lab, Shankar, Neema, Ryan, and Jessica, for their cooperation, and the positive work environment they have created.

I am grateful to my family back home and in Canada. This work would not have been completed without their consistent support and encouragement. They have been patiently awaiting my achievements, and understanding my long absence from home. I hereby dedicate my thesis to my supervisor and my family.
# TABLE OF CONTENTS

APPROVAL/SIGNATURE PAGE ii  
DEDICATION iii  
ABSTRACT iv  
ACKNOWLEDGEMENTS v  
TABLE OF CONTENTS vi  
LIST OF TABLES viii  
LIST OF FIGURES ix  
LIST OF ABBREVIATIONS x  
INTRODUCTION 1  
  General Information about Auxin 2  
    1. Auxin biosynthesis sites and pathways 3  
    2. Auxin transport pathways 4  
    3. PIN polarity determines the direction of auxin polar transport 5  
    4. Auxin response 6  
    5. Auxin canalization hypothesis 7  
Processes of Leaf Shape Establishment 9  
Processes of Vein Pattern Development 11  
Root Development and Gravitropism 13  
Objectives 15  
MATERIALS AND METHODS 17  
  Plant Materials 17  
  Growth Conditions 17  
  Mutant Phenotype Identification and Screening, Backcrossing and Segregation Analysis 18  
  Complementation Test 18  
  Phenotypic Analysis of Several Mutant Lines (Cotyledon, First and Fifth leaf, Shoot and Root) 19  
  Generation of Double Mutants 20  
  Introduction of DR5::GUS Transgene and Histochemical Staining for GUS 21  
  Mapping of Mutant Genes 22  
  Materials 23  
  Microscopy, Imaging and Statistics 23  
RESULTS 25  
  Genetic Analysis of 115-11-21-4, 31-83-4-3, 32-2-4 and 33-7-5(4) 25
Complementation Test of 115-11-21-4 to fkd1 and sfc 26
First Leaf Shape Analysis of 31-83-4-3, 32-2-4 and 33-7-5(4) 27
First Leaf Vein Pattern Analysis of 31-83-4-3, 32-2-4 and 33-7-5(4) 28
Cotyledon Vein Pattern Analysis of 31-83-4-3, 32-2-4 and 33-7-5(4) 29
Shoot Phenotype Analysis of 31-83-4-3, 32-2-4 and 33-7-5(4) 30
Root Phenotype Analysis of 31-83-4-3, 32-2-4 and 33-7-5(4) 31
DR5::GUS Expression is Reduced in 33-7-5(4) 32
Double Mutants cuc2-3 32-2-4 has Smooth Leaf Margin 33
Mapping of 32-2-4 and 33-7-5(4) 33
DISCUSSION
  31-83-4-3 Phenotype was Consistent with Defects in Auxin Transport 35
  Serrated Leaf of 32-2-4 May Result from Higher Auxin in the Leaf Margin 37
  Auxin Response is Reduced in 33-7-5(4) 39
  Conclusions 40
REFERENCES 42
APPENDIX 1. Selected mutant lines with leaf shape and/or vein pattern defects 71
**LIST OF TABLES**

Table 1. SSLP markers designed and used in mapping 32-2-4 and 33-7-5(4) .......................... 49
Table 2. Segregation analysis of various mutant families ...................................................... 51
Table 3. Complementation data of 115-11-21-4 ................................................................. 52
Table 4. First leaf shape characters of various genotypes at 21 DAG .................................. 53
Table 5. First leaf vascular pattern characters of various genotypes at 21 DAG .................. 54
Table 6. Cotyledon vascular pattern characters of various genotypes at 14 DAG .................. 55
Table 7. Adult plant shoot characters of various genotypes at 35 DAG .............................. 56
Table 8. Root characters of various genotypes from 4 DAG to 5 DAG ............................... 57
Table 9. Fifth leaf serrations numbers of various genotypes at 35 DAG ............................. 58
Table 10. Mapping distances between the mutations and the primers ................................. 59
LIST OF FIGURES

Figure 1. Diagrams of different leaf shape and leaf characters 60
Figure 2. Models of leaf shape and vein pattern formation 61
Figure 3. Complementation test of mutant 115-11-21-4 to fkd1 and sfc 62
Figure 4. Cleared first leaves of various genotypes viewed under dissecting light microscope 63
Figure 5. Cleared cotyledons of various genotypes viewed under dissecting light microscope 64
Figure 6. Shoot phenotype of various genotypes at 35 DAG 65
Figure 7. Root tip bend after rotated 90° for 2, 4, 6 hours of various genotypes 66
Figure 8. DR5::GUS expression in 33-7-5(4) and wild type root tips and first leaves 67
Figure 9. Fifth leaf phenotypes of various genotypes at 35 DAG 68
Figure 10. PCR products defining molecular markers used in mapping 69
Figure 11. Models for first leaf shape and vein pattern formation associated with defective auxin regulation of three mutant lines 70
LIST OF ABBREVIATIONS

Genes and Protein Nomenclature

UPPERCASE ITALICS    wild type gene
lowercase italics    mutant allele
UPPERCASE NO ITALICS  wild type protein

Genes

ABCB       ATP BINDING CASSETTE SUBFAMILY B
APG10      ALBINO AND PALE GREEN10
ARE        AUXIN RESPONSE ELEMENT
ARF        AUXIN RESPONSE FACTOR
ARF-GAP    ADP RIBOSYLACTION FACTOR GTPASE ACTIVATION PROTEIN
ATHB8      ARABIDOPSIS THALIANA HOMEOBOX8
AUX1/LAX   AUXIN RESISTANT1/LIKE AUX1
BDL        BODENLOS
CUC2       CUP-SHAPED COTYLEDON2
CVP2       COTYLEDON VASCULAR PATTERN2
DR5        5 DIRECT REPEATS OF AN AUXIN RESPONSE ELEMENT
FKD1       FORKED1
GL1        GLABROUS1
GSY        GRASSY
LCD1       LOWER CELL DENSITY 1
MP         MONOPTEROS
PAC        PALE CRESS
PIN        PIN-FORMED
SFC        SCARFACE
SSM        SHORT STEM AND MIDRIB
TAA        Tryptophan Aminotransferase of Arabidopsis
TAR2       Tryptophan Aminotransferase Related 2
UNH        UNHINGED
VAM        VACUOLAR MORPHOLOGY
WEI8       WEAK ETHYLENE INSENSITIV 8
YUC        YUCCA
**Chemicals**

- **2,4-D**: 2,4-dichlorophenoxyacetic acid
- **DMF**: Dimethylformamide
- **EDTA**: Ethylenediaminetetraacetic acid
- **EMS**: ethyl methane sulfonate
- **GFP**: green fluorescent protein
- **GUS**: β-GLUCURONIDASE
- **IAA**: indole-3-acetic acid
- **IPA**: indole-3-pyruvic acid
- **NPA**: 1-Naphthylphtalamic acid
- **TBE**: Tris/Borate/EDTA
- **Triton X-100**: polyoxyethylene octyl phenyl ether
- **Trp**: Tryptophan
- **X-gluc**: 5-bromo-4-chloro-3-indolyglucuronide

**Terms**

- **AGI**: *Arabidopsis* Genome Initiative
- **AT**: *Arabidopsis thaliana*
- **BAC**: bacterial artificial chromosome
- **bc1**: backcross to wild type once
- **bc2**: backcross to wild type twice
- **bp**: base pair
- **Col**: Columbia ecotype
- **cM**: Centimorgan
- **DAG**: Days After Germination
- **DZ**: differentiation zone
- **ER**: endoplasmic reticulum
- **EZ**: elongation zone
- **F1**: First filial generation
- **F2**: Second filial generation
- **GM**: ground meristem
- **Het**: heterozygous
- **Ler**: Lansberg *erecta* ecotype
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>seed mutagenized with a mutagen</td>
</tr>
<tr>
<td>M2</td>
<td>self-pollinated seed from M1</td>
</tr>
<tr>
<td>MZ</td>
<td>meristematic zone</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>RAM</td>
<td>root apical meristem</td>
</tr>
<tr>
<td>SAM</td>
<td>shoot apical meristem</td>
</tr>
<tr>
<td>s.e.m</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SSLP</td>
<td>simple sequence length polymorphism</td>
</tr>
<tr>
<td>TAIR</td>
<td>The <em>Arabidopsis</em> Information Resource</td>
</tr>
</tbody>
</table>
INTRODUCTION

Optimum leaf shape and size are very important to plant success, as leaves are the most important photosynthetic organs of vascular plants. Leaf physiological functions are supported by several specialized cell types, such as paired guard cells in the epidermis for gas exchange, mesophyll cells for photosynthesis, and vascular cells for internal fluid and nutrient transport (Esau, 1965a). As a fundamental component of the plant body, the continuous vascular network provides not only mechanical strength but also the key role of transport: the vascular tissue xylem transports water and minerals, and phloem translocates dissolved photoassimilates efficiently (Esau, 1965b). Leaf morphogenesis corresponds closely with genetic controls and environmental factors (Tsukaya, 2005) and leaf morphology is often used to distinguish different plant species. Over the past two decades, the isolation of leaf morphological mutants of Arabidopsis thaliana has been commonly used to further genetic studies of leaf development (Scarpella et al., 2010).

The leaf form of Arabidopsis thaliana is called a simple leaf (Figure 1A) and may have serrations (tooth-like projections; Figure 1B) and lobes (marginal segmentation; Figure 1C) to different extents along the leaf marginal area. In contrast, compound leaves have gaps that separate the single leaf blade into distinct units which are called lateral leaflets (Figure 1D) (Scarpella et al., 2010). The wild type Arabidopsis thaliana has a relatively complex vein pattern with closed vein loops (Nelson and Dengler, 1997): in the middle of the leaf blade is the midvein (primary vein) which is generally thicker than lateral veins; the veins connected to the midvein are called secondary veins, similarly the veins connected to secondary veins but not the midvein are called tertiary veins. Veins which end freely in the leaf marginal area are sometimes connected to hydathodes (Figure 1E). A
hydathode is an epidermal structure that is connected to a vein ending and has probably evolved from modified stomata. Hydathodes are specialized for secreting water, and are typically located at the leaf tip or the serration tip (Esau, 1965a).

Although there are detailed descriptions and consistent theoretical models to account for the ontogeny of leaf vascular patterns (Meinhardt, 1976, Mitchison, 1980), not all mechanisms of the patterning processes required to build such structures are known at the molecular level (Scarpella et al., 2004, Sack and Scoffoni, 2013). The auxin canalization hypothesis (Sachs, 1981) seems to best account for the continuous vascular strands and the final vein pattern. A positive feedback mechanism causes cell to cell auxin transport (both influx to and efflux from cells) to become more efficient; this results in stable "auxin canals". The increased conductivity of these cells is proposed to not only lead to their vascular differentiation (caused by the high level of auxin flux), but also to deplete neighboring cells of auxin preventing them from taking on a vascular cell fate (Sachs, 1981).

**General Information about Auxin**

Plant hormones are signaling molecules that act as growth regulators to control complex growth and developmental processes and often allow plants to respond to environmental changes (Berleth et al., 2004). Auxin is one of the most important plant hormones and plays a key role in many aspects of plant growth and development, including cell division and elongation, differentiation, patterning, tropisms, apical dominance, and flowering (Berleth et al., 2004). To understand the mechanism by which auxin controls
these processes, it is important to understand auxin regulation at three levels: auxin biosynthesis, auxin transport and auxin response.

1. Auxin biosynthesis sites and pathways

Auxin is synthesized in young tissues of both shoots and roots, but most auxin is synthesized in the shoot apex and young leaves (Ljung et al., 2001). The first identified plant hormone, indole-3-acetic acid (IAA), has been recognized as the major auxin for more than seventy years (Mashiguchi et al., 2011). Several auxin biosynthetic pathways have been reported recently, although the pathway by which auxin is synthesized in plants is still unclear at the genetic level (Mano and Nemoto, 2012). Tryptophan (Trp)-dependent and Trp-independent pathways have been proposed as the two major IAA biosynthetic pathways. There are four presumptive pathways in Trp-dependent IAA biosynthesis (Mano and Nemoto, 2012), and the indole-3-pyruvic acid (IPA) pathway is the one considered to be the main IAA biosynthesis pathway in Arabidopsis thaliana (Mashiguchi et al., 2011). Mashiguchi et al (2011) demonstrated that two enzymes, Tryptophan Aminotransferase of Arabidopsis (TAA) and YUCCA (YUC) flavin monooxygenase-like protein, are both required as they function in the same IPA pathway: the TAA family mainly produces IPA from Trp and the YUC family is mainly implicated in the conversion of IPA to IAA; thus YUC proteins catalyze a rate-limiting step of the IPA pathway that produces IAA that is essential for plant development.

The Arabidopsis thaliana genome contains 11 YUC gene members (YUC1-YUC11) that are mainly expressed in meristems, young primordia, vascular tissues, and reproductive organs. Within leaves, the expression of these genes is associated with leaf
margin and hydathode development (Wang et al., 2011). The YUC proteins appear to have overlapping functions; overexpression of each YUC gene leads to auxin overproduction, but disruption of a single YUC gene does not cause obvious developmental defects. Removal at least four genes (YUC1, YUC2, YUC4 and YUC6) results in a narrow leaf with leaf margin defects (Cheng et al., 2006, Cheng et al., 2007). The YUC and the TAA gene families have been proposed to play essential roles in auxin biosynthesis, and it has been suggested that they participate in two independent pathways. Study of taa mutant phenotypes has allowed assessment of the genetic interaction between YUC and TAA (Won et al., 2011). Mutations in TAA genes mimicked the phenotypes of yuc mutants and the yuc1 yuc4 wei8 tar2 quadruple mutants did not make any hypocotyls and roots (wei8 and tar2 are alleles of taa1), a phenotype that was not observed in either yuc1 yuc4 or wei8 tar2-1 double mutants (Won et al., 2011). Together, these phenotypes suggest that the YUC and TAA act in independent and redundant pathways of auxin biosynthesis.

2. Auxin transport pathways

Since most auxin is synthesized in the shoot apex and young leaves, auxin transport from the sites of synthesis (source tissue) to the sites of action (sink tissue such as the roots) is very important for development of the whole plant (Ljung et al., 2001). Generally auxin is transported in two major pathways. Most auxin is transported by bulk flow from source (young leaves) to sink (roots) through the mature phloem which plays the role of transporting metabolites from source (leaves) to the other parts of plant; this pathway is considered long distance transport (Petrášek and Friml, 2009). Another pathway is auxin transport from cell to cell in polarized streams via auxin influx and efflux carriers; this
pathway is considered short distance transport, and is facilitated by chemiosmotic gradients (Peer et al., 2011). When the extracellular auxin level is high, auxin can enter cells via lipophilic diffusion. In contrast, when the auxin level is low in the extracellular environment, auxin uptake from the environment to cells is mediated by the transmembrane proteins AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX) family which act as auxin influx carriers to create an auxin sink in cells (Bennett et al., 1996, Swarup et al., 2001). However, auxin is anionic in the cytosol (neutral pH) and cannot go passively through the membrane; instead it requires transporters to exit cells (Peer et al., 2011). Auxin efflux carriers, PIN-FORMED (PIN) proteins, mediate auxin export, and the ATP Binding Cassette subfamily B (ABCB) transporters can coordinate with PIN proteins in exporting auxin through the cell membrane (Titapiwatanakun et al., 2009, Zhang et al., 2011).

3. PIN polarity determines the direction of auxin polar transport

The Arabidopsis thaliana PIN family consists of eight gene members. Five of them encode full length plasma membrane (PM) localized PINs (PIN1, 2, 3, 4, and 7) that act as auxin efflux carriers (Petrasek et al., 2006), the other three genes encode short length endoplasmic reticulum (ER) localized PINs (PIN5, 6, and 8) that function in homeostatic auxin compartmentalization (Mravec et al., 2009). The PIN proteins have been identified and characterized as key regulators of auxin dependent processes, including axis formation in pre-and post-embryogenesis (PIN1, 4, and 7), root meristem maintenance (PIN1, 3, 4, and 7) (Feraru and Friml, 2008) and vascular tissue differentiation and regeneration (PIN1, 5, 6, and 8) (Sawchuk et al., 2013). PIN1 and PIN2 exhibit primarily polar localization on the PM. The polar localization at the single cell level determines the direction of
intercellular auxin transport and the direction of auxin transport between neighboring cells. For example, the apical localization of PIN1 results in upward auxin transport, while the basal localization of PIN1 results in downward auxin transport (Wisniewska et al., 2006). The fusion protein PIN1:GFP (green fluorescent protein) has been used widely to monitor the expression and localization of PIN1 in leaf cells. PIN1 localization can define the formation sites of not only serrations (or lobes), but also the veins, and the marker PIN1:GFP allows a dynamic observation of leaf shape and vein pattern developments to occur (Wenzel et al., 2007). In the wild type Arabidopsis thaliana PIN1:GFP expression extends from the epidermal cells of leaf primordia into the ground tissue. Within the first leaves, from 2 DAG (Days After Germination) old primordia onwards, PIN1 expression narrows from successive regions (several cells wide) within the ground meristem (GM) to files of cells (one to three cells wide) that form successive vein orders (Scarpella et al., 2006, Wenzel et al., 2007). The narrowing of PIN1:GFP provides visual evidence of the auxin canalization process (Wenzel et al., 2007).

4. Auxin response

Together with auxin synthesis and transport, proper auxin response is also important in plant development. Auxin Response Factors (ARFs) are DNA binding proteins that bind to Auxin Response Elements (AREs) and function as either activators or repressors that can either positively or negatively influence gene expression (Ulmasov et al., 1999, Tiwari et al., 2003). For example, ARF5, also known as MONOPTEROS (MP), is activated by auxin and induces the expression of auxin inducible genes such as PIN1 and ARABIDOPSIS THALIANA HOMEobox8 (ATHB8). By using in situ RNA hybridization,
MP expression can be analyzed at the transcript level. This technique has revealed that during leaf development, MP is initially expressed in broad domains that gradually become confined within the vascular tissues (Wenzel et al., 2007). MP can directly and positively regulate the expression of ATHB8 through an Auxin Response Element in the ATHB8 promoter (Donner et al., 2010). ATHB8 is expressed in both shoot and root vascular bundles, showing that it is correlated with vascularization throughout the plant, and the effects of ATHB8 on vein formation depend on the activity of MP (Baima et al., 2001). Within the broad field of cells expressing the Auxin Response Factor MP, only a subset of cells initiate ATHB8 expression (Baima et al., 1995).

5. Auxin canalization hypothesis

The auxin dependent vascular patterning model that is called the auxin canalization hypothesis is the most accepted model of vascular tissue formation: auxin creates a pathway for efficient auxin flow much like water eroding river beds; the auxin flow causes columns of cells to differentiate into procambium (vascular meristematic tissue) and eventually vascular tissue (Sachs, 1981). It has been proposed that auxin is initially produced in a broad region, then narrows to a file of cells; the presence of auxin in undifferentiated cells promotes cellular changes that allow auxin movement to change from slow diffusion (environment to cell, cell to cell via influx and efflux carriers) to rapid auxin flow (source to sink via mature phloem). The procambial cells appear as narrow, cytoplasmic-dense cells that emerge from the subepidermal tissue of the leaf primordium, termed the ground meristem (GM) (Foster, 1952, Esau, 1965b). Procambial cells are
characteristically arranged in continuous strands and acquire their narrow shape through coordinated, oriented divisions that are parallel to the axis of the emerging strand.

Recent studies provide more evidence that auxin flow is the primary regulator of vascular tissue formation. For example, either genetic inhibition (mutants) or chemical inhibition of auxin transport by 1-Naphthylphtalamic acid (NPA, a specific inhibitor of the auxin efflux) can result in various vein defects (Mattsson et al., 1999, Taiz and Zeiger, 2002). Based on the study of Mattsson et al. (1999), Arabidopsis thaliana pin1 mutants have additional primary and secondary veins, as well as abundant continuous veins along the leaf margin area. Inhibition of auxin transport by low concentration of NPA in wild type Arabidopsis thaliana can produce very similar vein features to that of pin1. The phenotype suggested that the leaf margin is a source of auxin for the GM. In the absence of auxin transport, auxin accumulates at the margin, resulting in vascular proliferation (Mattsson et al., 1999). This idea has been validated by the finding that PIN1:GFP is initially expressed in the marginal epidermis, where it generates auxin maxima that initiate PIN1 expression and vein formation within the GM (Scarpella et al., 2006, Wenzel et al., 2007).

Additional evidence for the importance of auxin distribution to vein formation comes from insensitive auxin response Arabidopsis thaliana mutants such as mp. The mp mutants are rootless and form fewer secondary veins with no veins along the leaf margin in both cotyledon and later leaves (Hardtke and Berleth, 1998). Following treatment with synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), MP transcription is induced by auxin and its expression pattern is similar to the pattern of auxin responsive reporter gene expression (Wenzel et al., 2007). Whereas early in mp mutant leaf development, the MP
expression is similar to wild type, in more advanced primordia MP expression is much reduced when compared to wild type accounting for the lack of higher-order veins in mp mutants leaves (Wenzel et al., 2007). In addition, by introducing the reporter transgene pPIN1::PIN1:GFP into the mp mutant background, it is validated that PIN1 expression is regulated by MP: in contrast to the normal MP expression in early mp mutant leaf development, PIN1:GFP expression is much reduced in mp mutants (Wenzel et al., 2007). Together, these data suggest a feedback loop consistent with the original canalization hypothesis: auxin induces MP, resulting in higher auxin response, which in turn induces PIN1, resulting in higher auxin transport and higher auxin in cells (Wenzel et al., 2007).

**Processes of Leaf Shape Establishment**

The interaction between the developing leaf primordia and the shoot apical meristem (SAM) is important in axis specification and phyllotaxis establishment, and is correlated with auxin transport direction and auxin activity (Figure 2 A). During the early stages of leaf development, apical PIN1 polarity in the SAM epidermal cells directs auxin flow toward the flanks of the SAM (Scarpella et al., 2010). A region of high auxin activity (epidermal PIN1 convergence point) is created that defines the site of the initial leaf primordium (Figure 2 B). During leaf formation, the establishment of three axes plays an important role in the shape change of the initial leaf primordia and appears to be coupled with the flat structure of leaf blade or leaf lamina (Waites and Hudson, 1995). These three axes are the proximal-distal axis, the medial-lateral axis and the adaxial (upper side which faces the SAM)-abaxial (lower side which opposes the adaxial side) axis (Scarpella et al., 2010).
The basic leaf shape is formed during primary morphogenesis, a stage in which cell division is predominantly responsible for growth. In the process of leaf lamina expansion, a short-lived marginal meristem is activated along the edge of the adaxial/abaxial boundary of the initial leaf primordium; leaf lamina expansion proliferates from the marginal meristem and converts the leaf primordium from rod shaped to spatula shaped (Donnelly et al., 1999). Following this process, in the inner area of leaf primordia, the internal meristem is activated to establish the inside structure of the leaf blade: the cells at the adaxial side differentiate into palisade cells and the cells at the abaxial side differentiate into spongy cells in most plant species (Donnelly et al., 1999). During the expansion of the leaf blade, cell proliferation is also controlled along proximal-distal and medial-lateral axes (Tsuge et al., 1996). In Arabidopsis thaliana leaves, the proximal-distal and medial-lateral polarities are important in determining leaf length and width respectively, and the proximal-distal and medial-lateral axes are correlated with the final shape: the distal part of leaf primordium differentiates into the leaf blade while the proximal part of the leaf primordium develops into the leaf petiole (Tsukaya, 2005).

Serrations and lobes are formed along the leaf margin during secondary morphogenesis, a stage in which cell expansion is predominately responsible for growth (Scarpella et al., 2010). Very similar to the initiation sites of leaf primordia on the SAM, elevated auxin activities in the leaf marginal area correspond to epidermal PIN1 convergence points that predict initiation of serrations (or lobes) at later stages of leaf development (Figure 2 C) (Hay et al., 2006, Scarpella et al., 2006). The operation of auxin activity maxima requires three processes. The first process is the polar (apical or basal) localization of PIN1 that determines the direction of auxin transport (red arrows in Figure
2 D); the second process is auxin flow that enhances PIN1 localization with the same polarity (black arrows in Figure 2 D). Together, these two processes create the maxima and minima of auxin concentration. The third process is the expression of CUP-SHAPED COTYLEDON2 (CUC2) transcription factor in the sinuses between serrations, where it is proposed to repress growth (Nikovics et al., 2006). CUC2 also enables reorientation of PIN1 (dashed black arrows in Figure 2 D) and, in turn, auxin inhibits CUC2, thus stabilizing the position of auxin maxima/CUC2 minima (predicting serrations) and auxin minima/CUC2 maxima (predicting sinuses) (Bilsborough et al., 2011).

**Processes of Vein Pattern Development**

The basic leaf vasculature pattern is also formed during the primary morphogenesis process. During leaf lamina expansion, the concentrated auxin at the tip of the primordium is transported from the tip into the GM through basal PIN1 polarity in subepidermal cells (Benkova et al., 2003, Scarpella et al., 2006); the auxin flow through the leaf centre marks the position of the primary midvein (Figure 2B). In addition, the basal PIN1 polarity directs the lateral veins (secondary veins) to differentiate from the leaf margin toward the midvein (Figure 2C); subsequently, the bipolarity of PIN1 within specific cells can establish the closed vein loop later in vasculature development process (Scarpella et al., 2006, Wenzel et al., 2007, Scarpella et al., 2010). Dual PIN1 polarity is required in the lateral vein formation to establish closed vein loops: the basal PIN1 localization directs auxin transport from the leaf marginal area to the proximal midvein which induces the lateral veins to form toward the proximal midvein; once the veins become connected, the PIN1 localization becomes apical and directs auxin transport from the lower part of lateral veins to the distal
midvein. Thus the upper part of lateral veins extend from the lower part toward the distal midvein and finally form closed vein loops (Figure 2G) (Wenzel et al., 2007, Scarpella et al., 2010). Open-ended marginal vein precursors grow in both directions toward the pre-existing lateral veins, either forming closed vein loops or ending freely (Scarpella et al., 2010). During secondary morphogenesis, similar to marginal veins, higher order veins (quaternary veins) appear in continuity within the expanding lamina and either end freely or connect to the pre-existing veins (Scarpella et al., 2010).

It has frequently been observed that the branched venation pattern largely reflects the leaf shape, such as veins extending into the marginal area at the leaf serrations in *Arabidopsis thaliana* leaves (Nelson and Dengler, 1997, Dengler and Kang, 2001). One explanation is that the formation sites of veins from the leaf marginal area and the positions of the serrations are both correlated with PIN1 convergence within the marginal epidermis (Bilsborough et al., 2011, Pahari et al., 2014). *Arabidopsis thaliana* mutants with altered PIN1 marginal expression have altered leaf vein and serration phenotypes (Shirakawa et al., 2009, Pahari et al., 2014). For example, *unhinged-1 (unh1)* leaves have serrated leaf margins combined with a simpler leaf vein pattern and frequent distal free-ending veins (Figure 2 H) (Pahari et al., 2014). This phenotype results from expanded PIN1 expression in the marginal area; the predicted elevated auxin activities are proposed to lead to marginal out-growth (here represented as serrations) with free-ending lateral veins (Pahari et al., 2014).

Several other mutant *Arabidopsis thaliana* have abnormal vein patterns which result from defects related to PIN1 expression and/or polar localization. The *Arabidopsis thaliana* mutant *forked1 (fkd1)* leaves have normal shape but frequent distal free-ending
veins (Steynen and Schultz, 2003). Previous studies have determined that \textit{FKD1} encodes a protein that is required for PIN1 localization in developing leaf veins (Hou \textit{et al.}, 2010). The absence of FKD1 delays the narrowing process of PIN1:GFP expression within incipient veins. As well, PIN1:GFP localization at the apical cell face is infrequent, which correlates with the failure of newly forming veins to connect to the previously formed veins. The \textit{scarface (sfc)} mutant plants also have normal leaf shape but like \textit{fkdl}, \textit{sfc} has frequent free-ending veins as well as small vein segments called vascular islands, that do not connect to other veins (Deyholos \textit{et al.}, 2000). Previous studies have determined that \textit{SFC} encodes an ADP ribosylation factor GTPase activation protein (ARF-GAP) that is required for normal auxin efflux and vein patterning (Sieburth \textit{et al.}, 2006) through appropriate PIN1:GFP localization (Scarpella \textit{et al.}, 2006). Although proper PIN1 expression and polar localization are essential in auxin dependent vascular formation, \textit{Arabidopsis thaliana pin1} mutant leaves have relatively mild vein-pattern defects when compared with \textit{fkdl}, \textit{sfc} and \textit{unh}, indicating that the redundancy of other PIN proteins (PIN5, 6, and 8) in vein patterning underlies the developmental processes. Thus, when there is defect to one or two PIN protein expression or localization, the mutant plant may still be able to generate a relatively normal phenotype because the other PIN family members allow proper growth and development processes (Sawchuk \textit{et al.}, 2013).

**Root Development and Gravitropism**

Auxin plays an important role in the embryonic root formation and root growth regulation. Different auxin concentrations have bimodal effects on primary root length (Eliasson \textit{et al.}, 1989), and also control the root response to gravity (gravitropism)
Auxin is synthesized in shoots (young leaves and cotyledons) and can be transported in a polar fashion from source to the sink such as roots (Ljung et al., 2002). The downward auxin transport from shoot tissues to the root and auxin redistribution in the root tip are essential for root development and gravitropism. In addition to being synthesized in the shoots, auxin is also synthesized in the root, and root-generated auxin contributes to the maintenance of the auxin gradients which is required for normal root development (Ljung et al., 2005). The local auxin concentration gradients and maximum auxin activity sites are crucial for primary root cell division and elongation.

During root embryogenesis, the root apical meristem (RAM) is established that will later provide new cells for the growing root. After the patterning process of RAM embryogenesis, the post-germination primary root matures through three distinct developmental zones. In the meristematic zone (MZ), a pool of cells is generated and will elongate and differentiate; once cells enter the elongation zone (EZ), cell division rate slows and cell expansion begins, the length of the cells will increase by many times their width; when cells in EZ reach their final size, they enter the differentiation zone (DZ), where the cells acquire their specialized characteristics and functions such as the formation of root hairs (Petricka et al., 2012). The effect of auxin on primary root growth is based on both the cell division rate that occurs in the meristematic zone, and the cell expansion and elongation that occurs in the elongation zone (Overvoorde et al., 2010).

Auxin is one of the key regulators of cell division in the MZ and elongation in the EZ that together contribute to primary root growth. Recent studies have suggested an auxin reflux loop that generates an auxin gradient in the root tip and maintains an auxin maximum in the RAM: auxin is first transported by PIN1, PIN2, PIN3 and PIN4 from the shoot
through root vasculature and then redistributed in the root cap to the external root cells; auxin is then transported back toward the shoot by PIN2, PIN3 and PIN7 in the external root layers (Petricka et al., 2012, Geisler et al., 2013).

Root gravitropism is a complex process that allows the root to grow downward into the soil. The Cholodny-Went hypothesis suggested the root bending is caused by altered redistribution of auxin during root gravitropism (Went, 1974). During the last three decades, evidence has grown that cell-to-cell or polar auxin transport is sufficient to generate differential intercellular auxin gradients that guide root growth (Rashotte et al., 2000). Following a 90° turn of the vertically grown roots, the auxin is redistributed to the lower side of the root tip within minutes due to the relocalization of PIN3; this results in asymmetric accumulation of auxin on the lower side of the root which inhibits cell elongation; meanwhile the cells at the upper side of the root continue to elongate which bends the root tip to growth downward again (Petricka et al., 2012). As auxin is normally required to maintain cell elongation in the EZ, the redistribution of auxin toward the lower side of the root tip maintains these cells in this area in a proper dividing and elongating status (Petricka et al., 2012).

Objectives

Although a vast inventory of morphological mutants of *Arabidopsis thaliana* is available, only some have been used for genetic studies of leaf development (Serrano-Cartagena et al., 1999). In this thesis, with the aim of contributing to the genetic analysis of leaf development and in particular the coordination of leaf shape and vein pattern by auxin, I focused on mutants that appeared to have abnormal shape (elongated narrow leaves
with/without obvious serrations) and leaf venation defects (frequent free-ending veins, lower vein density). Twenty-four ethyl methane sulfonate (EMS, which induces random mutations by nucleotide substitution) mutagenized mutant lines of Arabidopsis thaliana in Columbia ecotype were chosen as candidates for mutations in genes that are required for leaf morphogenesis, and these mutant lines were further characterized by assessing the first leaf phenotypes.

I hypothesized that a mutation in a single gene with a critical role in auxin regulation could change the whole plant development and alter the whole plant phenotype. With this hypothesis, the first leaf phenotype, the cotyledon phenotype, other shoot and root characters such as flowering time, stem branching, number of rosette leaves, root elongation, and gravitropism were also quantified to determine if global changes in auxin regulation were occurring in the mutants.
MATERIALS AND METHODS

Plant Materials

Wild type *Arabidopsis thaliana* seeds (Columbia ecotype) mutagenized with ethyl methane sulfonate (EMS) were purchased from Lehle Seed (Round Rock, TX). The *cup-shaped cotyledon* (*cuc2-3*) seeds of Columbia ecotype (Col) were generously provided by Masao Tasaka and Mitsuhiro Aida (Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara, Japan), and *DR5::GUS* by Jane Murfett (University of Missouri, Columbia, MO). All other seed material was generated in the lab. Wild type *Arabidopsis thaliana* of Col ecotype was used as a control for all the experiments.

Growth Conditions

Seeds were planted either on a mixture of ¾ Potting Mix and ¼ vermiculite (both from Coaldale Nurseries, Coaldale, AB) in 100 cm² pots or on *Arabidopsis thaliana* (AT) growth medium (Ruegger et al., 1998) in Petri dishes. Pots were covered with plastic wrap and both pots and plates were incubated at 4°C in the dark for 3 days to allow most seeds to germinate synchronously, after which they were transferred to growth chambers (Percival Scientific, Perry, IA) with 24 hours of continuous light at a photon flux density of approximately 130 μmol s⁻¹ m⁻² from Sylvania Cool White, Grow Lux, and incandescent bulbs (Osram Sylvania Inc, Danvers, MA). Chambers were set at 22°C and 60% relative humidity. The day of transfer to the growth chambers was considered to be the day of germination or 0 Days After Germination (DAG). Plastic wrap was removed at 7 DAG and plants were maintained under constant growth conditions.
Mutant Phenotype Identification and Screening, Backcrossing and Segregation Analysis

Seeds mutagenized with EMS (Lehle Seed, Round Rock, TX) in Col ecotype are known as the M1 generation, its self-pollinated progeny, is the M2 generation, and so on. The mutant lines that are used in this thesis had been previously identified at the M2, M3, and M4 generations; the M4 seeds were planted and screened for abnormal first leaf defects by using wild type Columbia ecotype as a control for phenotypic comparisons. Mutations in M4 generation were backcrossed to the wild type, the F2 generation of backcrossing were examined for plants with first leaf shape and/or vein pattern defects, as well as the segregation ratio. For this study, either self-pollinated progeny from the EMS mutagenized mutants (e.g. M4) or progeny selected in the segregating population of the mutants which had been crossed to wild type (Col) (e.g. bc1F2) were screened. To determine the segregation ratio, these seeds were sown at a density of about 25 seeds per pot and 21 DAG first rosette leaves were cleared in cytoseal for identification of mutant phenotypes. In total, twenty-four mutant lines with either leaf shape defects or leaf vein pattern defects, or both, have been selected for segregation analysis and further phenotype analysis (see Appendix 1 for details). The Pearson’s chi-squared test was used to establish that the mutant alleles segregated as expected for a single recessive mutation (3:1).

Complementation Test

Plants with selected single recessive mutations identified in this study were crossed to the previously identified mutants (e.g. fkd1, sfc, unh, gsy) that were available in our laboratory for complementation tests based on the phenotype. To be certain that the F1
seeds were from outcrossing, not from self-fertilization, a female glabrous1 (gl1, hairless) plant of a mutant selected in this study was crossed to a male GLABROUS1 (GL1, with hair) plant of a previously identified mutant (Larkin et al., 1994). Since GL1 is dominant, all the outcross F1 population plants will have hairs. If the plants of the F1 generation are all mutant phenotype, then the female mutant line and the male mutant line are allelic. In contrast, if the F1 generation are all wild type phenotype, then the female mutant line and the male mutant line are not allelic.

Phenotypic Analysis of Several Mutant Lines (Cotyledon, First and Fifth leaf, Shoot and Root)

To analyze and compare the cotyledon, first and fifth rosette leaves of all genotypes, homozygous mutant seeds (31-83-4-3 from bc3F3, 32-2-4 from bc4F3, and 33-7-5(4) from bc4F3) and Col seeds were sown on soil at a density of 16 seeds per pot; from the two leaved stage the density was maintained at 12 healthy plants per pot. For all genotypes except 33-7-5(4) (seven days later than the normal DAG), cotyledons were removed at 14 DAG, first leaves were removed at 21 DAG, and fifth leaves were removed at 35 DAG, then treated with 70% ethanol for 1 day and cleared in chloral hydrate (Sigma): water: glycerol (8:2:1v/v/v) (Koizumi et al., 2000) for one week prior to mounting in glycerol: water (2:1).

In the analysis of cotyledon and first leaf vein patterns, the mid-vein (primary vein) was considered to be the linear vascular strand approximately along the midline, secondary veins were considered to be those vascular strands connected to the mid-vein, tertiary veins were the veins connected to secondary veins (but not the mid-vein) and quaternary veins
are the veins connected to tertiary veins (but not the mid-vein or secondary veins). These vascular strands were identified based on differentiated xylem. Branch points (junction of 2 veins meeting), areoles (area of leaf completely enclosed by veins), vascular islands (fragments of discontiguous vasculature), free-ending veins (veins connected at one end but disconnected at the other end), midvein extension (midvein ends freely at the tip and is not connected to secondary veins) and marginal venation gaps (a vascular discontinuity in the peripheral venation that creates a vein-free path to the mid-vein) were scored (Figure 1E).

In the analysis of first leaf shape, the leaf width (the longest line perpendicular to the mid-vein that extends from leaf margin to leaf margin), length and length/width, marginal early hydathodes, and serrations were also scored.

To analyze plant shoot morphology, plants were scored for flowering time when the inflorescence stem was 2 cm long, and the number of rosette leaves and stem branches were also recorded at 35 DAG.

To analyze root growth, seeds were sown on AT medium (Ruegger et al., 1998) at a density of 30 seedlings per plate and grown vertically. Primary root lengths were measured at 4 DAG and 5 DAG to determine the root elongation within 24 hours. Root gravitropic response was determined by rotating vertically grown 5 DAG seedlings 90°. Images of the root tip were captured at 2, 4, and 6 hours after rotation, and the angles of root tip bending were measured by using ImageJ (http://imagej.nih.gov/ij).

**Generation of Double Mutants**
To generate double mutants between each leaf shape or vein pattern mutant and\textit{cuc2}-3, homozygous mutant plants were crossed to \textit{cuc2}-3 plants to generate the F1 seeds. F1 plants were allowed to self-pollinate for F2 progeny. Seeds from F2 plants with the mutant leaf vein phenotype were harvested as F3. F3 populations were screened for segregation of \textit{cuc2}-3 (expecting lack of serrations). When the double mutant plants were fertile, seeds of double mutants segregating in the F3 were planted and the F4 plants were characterized. If double mutants were sterile (31-83-4-3), analysis was done in the segregating F3 population. All the double mutants were scored for total number of serrations on the fifth leaf of plants that were 35 DAG.

\textbf{Introduction of DR5::GUS Transgene and Histochemical Staining for GUS}

The \textit{DR5::GUS} transgene was introduced into each mutant by crossing mutant plants with homozygous \textit{DR5::GUS} plants in the Col ecotype to generate the F1 seeds. F1 plants were allowed to self-pollinate for F2 progeny. Seeds from F2 plants with the mutant leaf vein phenotype were harvested as F3. Expression of \textit{DR5::GUS} was screened in F3 populations; individual F3 populations that were all expressing indicate homozygosity for \textit{DR5::GUS} and were used for characterization. Mutants and wild type seed expressing \textit{DR5::GUS} were planted at a density of 30 seeds per plate on AT plates. GUS staining was performed after Kang and Dengler (2002). Briefly, seedlings were removed from the medium, placed in acetone on ice for 20 minutes and washed twice with 50 μM NaPO4 Buffer (pH 7). The seedlings were vacuum infiltrated for 10 minutes with GUS staining buffer [final concentration: 0.2% Triton X-100; 50mM NaHPO4 Buffer (pH7.2); 2mM Potassium Ferrocyanide; 2mM Potassium Ferricyanide; 2mM X-gluc, from 100mM stock
solution in DMF)] (Vitha et al., 1995) and then left for 16 hours before the GUS buffer was removed. Seedlings were decolourized with four rinses of 70% ethanol and finally cleared with chloral hydrate.

**Mapping of Mutant Genes**

Mapping was carried out through the use of ecotype-specific markers visible through PCR (simple sequence length polymorphisms, SSLPs) as described by Lukowitz et al. (2000). Once the phenotypes were determined to result from single nuclear recessive mutations, the homozygous mutant plants (32-2-4 from bc4F2 and 33-7-5(4) from bc3F2) were crossed to the Landsberg erecta (Ler) ecotype wild type and F2 seed was collected for the mapping population. DNA was extracted from the leaves of F2 plants showing the mutant phenotype (Dellaporta et al., 1983). Previously identified primers were obtained from Arabidopsis website TAIR (http://www.arabidopsis.org/index.jsp). Useful small insertions/deletions (INDELs for SSLPs) between the publicly available Col sequence and Ler sequence were generated through Monsanto Company (http://www.arabidopsis.org/browse/Cereon/index.jsp) (Jander et al., 2002). The web-based programs Primer3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and IDT OligoAnalyzer 3.1 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer) were used to locate and design primers around each polymorphism. Successful marker primers are listed in Table 1, as well as the amplified DNA product lengths for both Col and Ler ecotypes. PCR was done using standard protocol (Bell and Ecker, 1994) [final concentration: Standards Taq Reaction Buffer, 1X; Mg^{2+}, 2.5 mM (or as indicated in Table 1); dNTPs, 160 µM each;
primer, 0.05 μM each; DNA Template, extracted from young leaves, 1 to 2 μl per 20 μl reaction; Taq DNA Polymerase 0.025 U] for 32-40 cycles at the specific annealing temperature for each primer pair (Table 1). PCR products were resolved by gel electrophoresis using a 2% to 4% agarose gel in Tris/Borate/EDTA (TBE) Buffer at 70–80 V depending on the product size.

Materials

5-bromo-4-chloro-3-indolyglucuronide (X-gluc), purchased from Rose Scientific, Edmonton, AB. Ethidium bromide was purchased from Sigma Chemical Co (St. Louis, MO); dNTPs were purchased from Invitrogen (Burlington, ON); primers were synthesized by either Invitrogen (Carlsbad, CA) or Integrated DNA Technologies (Coralville, IA); Taq DNA Polymerase was purchased from Truin Scientific (Edmonton, AB). Agarose was purchased from Bioshop Canada Inc. (Burlington ON).

Microscopy, Imaging and Statistics

A Leica MZ8 dissecting light microscope (Leica Microsystems, Wetzlar, Germany) was used for leaf shape and venation analysis of mature cotyledons and leaves. Tissues were either photographed using a Nikon Coolpix 990 camera (Nikon, Mississauga, ON) or an AxioCam ICc1 digital microscope camera (for the tissues that were stained with GUS); and the pictures were scored using Paint.NET (http://www.getpaint.net) and ImageJ (http://imagej.nih.gov/ij).

Measurements were recorded in Microsoft Excel (Redmond, WA) for subsequent determinations of average and standard error of the mean (s.e.m). The Pearson’s chi-
squared test was used to determine the significance (p < 0.05) of segregation from backcrosses and the gene linkage from mapping. The value of the test-statistic is

\[ \chi^2 = \sum \frac{(O - E)^2}{E} \]

where \( \chi^2 \) = Pearson's cumulative test statistic, \( O \) = an observed frequency; \( E \) = an expected (theoretical) frequency; the approximate p value of \( \chi^2 \) was calculated using the online tool (http://www.socscistatistics.com/pvalues/chidistribution.aspx). The two-tailed Student's \( t \)-test assuming equal variance was applied in Microsoft Excel to determine the statistical differences for data sets with equal variance as determined by the \( F \)-test (p > 0.05). Data sets with unequal variance as determined by the \( F \)-test (p < 0.05) were analyzed by using two-tailed \( t \)-test assuming unequal variances. When a data set had no variation, the data points were converted from numerical to categorical to apply the two-tailed Fisher’s exact test using the online data analysis tool (http://graphpad.com/quickcalcs/contingency1).
RESULTS

The first leaves of wild type *Arabidopsis thaliana* (Col) have a spoon-shaped leaf blade, smooth leaf margins and a relatively closed vein pattern without many secondary free ending veins (Candela *et al.*, 1999). Some mutant *Arabidopsis thaliana* can generate abnormal leaves, such as narrow or curved leaf shapes, leaves with serrated leaf margins and leaves with simplified or frequent non-meeting vein patterns (Berná *et al.*, 1999). A total of twenty-four EMS mutagenized lines that had been previously identified as having defects of leaf shape and vein pattern were selected for further analysis (See Appendix 1 for details). After further backcrossing, only four mutant lines (31-83-4-3, 32-2-4, 33-7-5(4) and 115-11-21-4) showed stable phenotypes that segregated approximately 3:1 in the F2 generation. These mutant lines were subjected to more detailed genetic and phenotypic analysis.

Genetic Analysis of 115-11-21-4, 31-83-4-3, 32-2-4 and 33-7-5(4)

To determine the segregation ratio, self-fertilized seed as well as the F2 seeds of a backcross to each mutant line were sown on soil and the first leaves were cleared for phenotypic observation. For these four mutant lines, no wild type phenotype appeared within the self-fertilized populations, which means these four recessive mutations were all completely penetrant. For 32-2-4 and 115-11-21-4, segregation ratios of approximately 3:1 were observed in the F2 of the fourth and second backcross respectively (Table 2), indicating that these two mutant phenotypes result from single recessive mutations. For 31-83-4-3 and 33-7-5(4), segregation ratios of approximately 9:1 and 7:1 were observed in the F2 generation of the third and fourth backcross respectively (Table 2). The most likely
explanation for these lines with non 3:1 segregation ratios was that these two mutant phenotypes result from single recessive mutations (expect 15:1 ratio for recessive mutations in two genes), but that the mutations cause germination and seedling development defects. In addition, 31-83-4-3 may also cause gametophytic lethality, since the plants with the mutant phenotype were male sterile with infrequent pollen (data not shown). Line 33-7-5(4) may also cause embryonic lethality, since aborted seeds within developing siliques of backcross F1 plants were observed (data not shown).

To determine the germination rate and observe the seedling development at 4 DAG, homozygous seed were sown on AT plates. For the homozygous 31-83-4-3 population, the germination rate was 81% (n=57) and the seedling lethality rate was 48% (n=46). For the homozygous 33-7-5(4) population, the germination rate was 96% (n=55) and the seedling lethality rate was 85% (n=53). In contrast, the wild type germination rate is 100% (n=56) and no dead seedlings were observed. Thus, it seems possible that the deviation from a 3:1 ratio results from reduced germination and seedling lethality.

**Complementation Test of 115-11-21-4 to fkd1 and sfc**

From the initial screen, mutant 115-11-21-4 was observed to have elongated first leaf shape and frequently exhibited distal free ends of secondary and tertiary veins (Figure 3 A). Based on the similarity of first leaf phenotype to *forked1* (*fkd1*) (Figure 3 B) and *scarface* (*sfc*) (Figure 3 C) which have frequent distal free ending veins (Steynen and Schultz, 2003, Sieburth *et al.*, 2006), crosses between mutant 115-11-21-4 and *fkd1* or *sfc* were made for a complementation test (Table 3). The F1 generation of mutant 115-11-21-4 and *fkd1* were all mutant with the phenotype of frequent free ending veins in the first leaf
(Figure 3 E, n=62). The F1 generation of mutant 115-11-21-4 and sfc were all wild type phenotype (Figure 3 F, n=51). These data showed that mutant 115-11-21-4 was an allele of fkd1. Because the phenotypes of six fkd1 alleles have been previously published (Hou et al., 2010), and the phenotype of 115-11-21-4 was not obviously different to the previously identified fkd1, no further phenotypic analysis was done for this mutant line.

**First Leaf Shape Analysis of 31-83-4-3, 32-2-4 and 33-7-5(4)**

In order to determine the specific quantitative differences between mutant and wild type Arabidopsis thaliana first leaves, several leaf shape and vascular patterning characters were measured in mature first leaves. The first leaves of wild type, mutant 31-83-4-3 and 32-2-4 were removed at 21 DAG, while the first leaves of mutant 33-7-5(4) were removed at 28 DAG due to the slower growth rate.

Wild type Arabidopsis first leaves were shaped like spoons (leaf length/width=1.10±0.01, n=30) with a smooth leaf margin (no serrations), and generally have three hyathodes (Figure 4A, Table3). Mutant 31-83-4-3 first leaves were quite narrow and elongated (leaf length/width=1.52±0.03, n=30) with an obviously pointed leaf tip (Figure 4B, C), more pronounced serrations (1.60±0.16, n=30), and less pronounced hyathodes (1.63±0.14, n=30) (Table 4). Mutant 32-2-4 first leaves were slightly more elongated (leaf length/width=1.20±0.01, n=30) but a bit smaller than wild type (Figure 4D), with more pronounced serrations (1.70±0.15, n=30) and hyathodes (3.27±0.08, n=30) (Table3). Mutant 33-7-5(4) first leaves were more elongated (leaf length/width=1.31±0.04, n=30) but significantly smaller than wild type, and were flipper shaped with a very pointed
leaf tip (Figure 4E, F) with more pronounced serrations (1.87±0.16, n=30) and less pronounced hydathodes (2.60±0.14, n=30) (Table 4).

**First Leaf Vein Pattern Analysis of 31-83-4-3, 32-2-4 and 33-7-5(4)**

The venation pattern of wild type *Arabidopsis thaliana* first leaves is mostly predictable: a single primary vein (the midvein) runs through the middle of the leaf lamina from the stem to the distal leaf tip; the secondary veins branch from the distal part of the midvein on both sides and extend toward the margin, and either end freely (free-ending veins), or ultimately connect to other veins (the proximal part of the midvein, another secondary vein or higher-order veins); tertiary veins branch from secondary veins and quaternary veins branch from tertiary veins (Wenzel et al., 2007). In wild type most veins are connected to each other to form regions of the lamina that are completely enclosed by vein loops called areoles, and the junction of two veins is called a branch point (Dengler and Kang, 2001).

In 31-83-4-3 first leaves, the midvein frequently extends to the leaf tip and terminates in a hydathode which is termed a midvein extension (as shown in Figure 1 E, and Figure 4 C), while in wild type first leaves, the midvein never reaches the leaf tip, although a hydathode forms at the leaf tip (Table 5). Mutant 31-83-4-3 has a strong reduction in the number of secondary, tertiary and quaternary veins and a higher percentage of free-ending secondary veins, therefore the total number of branch points and areoles are reduced significantly. Vascular islands (a vein fragment that is disconnected to other veins) and marginal venation gaps (a vein-free path from leaf margin area to midvein) are observed frequently in 31-83-4-3 first leaves (Table 5, Figure 4 C).
Mutant 32-2-4 first leaves mimic the wild type vein pattern and are relatively predictable. The number of secondary, tertiary and quaternary veins is reduced relative to wild type, resulting in a reduction in the total number of areoles and branch points. The percentage of free-ending veins is quite similar to wild type. Like 31-83-4-3, some of the mutant 32-2-4 first leaves have vascular islands (Table 5).

Like 31-83-4-3, mutant 33-7-5(4) first leaves frequently have a midvein extension and marginal venation gaps. In addition a significant reduction in the number of secondary, tertiary and quaternary veins, and a higher percentage of free-ending secondary and tertiary veins occurs in this mutant. As a result of these characteristics, the total numbers of areoles and branch points are significantly reduced (Table 5).

**Cotyledon Vein Pattern Analysis of 31-83-4-3, 32-2-4 and 33-7-5(4)**

In addition to the first leaf vascular patterning characters, the vein pattern phenotype of wild type and three mutant lines was scored in mature cotyledons using the same rules as for first leaves. A variety of vascular pattern defects were found in mutant cotyledons, which were not seen in wild type cotyledons.

Wild type *Arabidopsis thaliana* cotyledons usually have two to four areoles formed by the closed secondary and tertiary vein loops (Figure 5 A-D). No free-ending proximal secondary veins were observed in wild type cotyledons, and quaternary veins were observed rarely and only in wild type (Table 6).

Generally, mutant 31-83-4-3 cotyledons have an increased percentage of distal and proximal free-ending secondary veins compared to wild type, resulting in a reduced number of areoles (Table 6). The frequent observation of midvein extension in 31-83-4-3
cotyledon (Figure 5 E, F, red arrows) is similar to that in the first leaves. In addition, some cotyledons have extremely abnormal vein patterns, with altered midvein location (Figure 5 G, H, dashed yellow lines indicate the normal midvein location).

Mutant 32-2-4 cotyledons have a vein pattern quite similar to wild type (Figure 5 I-L), with the numbers of areoles, branch points, and free-ending veins not significantly different from wild type (Table 6). In addition, 8.33% (n=48) of cotyledons have closed tertiary vein loop (tertiary vein branches from secondary vein and connected to the same secondary vein, indicated by the red arrows in Figure 5 J), instead of the normally closed secondary vein loop (secondary vein branches from secondary vein but connected to the midvein) in wild type.

Mutant 33-7-5(4) cotyledons have a significant reduction in the number of secondary and tertiary veins, and an increased percentage of distal and proximal free-ending secondary veins (Table 6). Therefore the total number of areoles and branch points is reduced in 33-7-5(4) cotyledons. Some cotyledons have a very simple vein pattern, such as only a midvein (Figure 5 P).

**Shoot Phenotype Analysis of 31-83-4-3, 32-2-4 and 33-7-5(4)**

The range of defects to first leaf shape and vein pattern, as well as the cotyledon vein pattern described above are often correlated with defects to auxin biosynthesis, transport and response. To determine if the mutations affected auxin regulated shoot developmental processes, shoot characters such as number of rosette leaves, number of stem branches, and flowering time were measured and compared among wild type and the three mutant lines.
The average flowering time of wild type *Arabidopsis thaliana* was about 21 DAG, and at 35 DAG the wild type plants had about seven rosette leaves and three to four lateral inflorescence stems; the main stem was longer than lateral inflorescence stems (Figure 6 A, Table 7).

The average flowering time of mutant 31-83-4-3 plants was about 29 DAG which was significantly delayed compared to wild type (Table 7). At 35 DAG, the 31-83-4-3 plants had about nine rosette leaves and seven inflorescence stems, but the rosette leaves were smaller and main inflorescence stem was shorter than wild type. The inflorescence changes resulted in 31-83-4-3 plants having a bushy appearance. A unique shoot character, fused leaf and stem (Figure 6 B, white squares) was frequently seen in this mutant (54.17%, n=24), as well as fused leaves, fused sepals, curved petals and improperly developed pistils and stamens (data not shown).

The flowering time of 32-2-4 was about two days later than wild type, and 32-2-4 plants had more leaves and fewer branches (Figure 6 C, Table 7).

Mutant 33-7-5(4) plants were pale and grew significantly more slowly than the wild type and other two mutant lines (Figure 6). They produced very small weak leaves and inflorescence stems; the average flowering time was more than 30 DAG.

**Root Phenotype Analysis of 31-83-4-3, 32-2-4 and 33-7-5(4)**

A certain auxin concentration, proper auxin transport and response are required for the normal root elongation and gravity response. To determine if the mutations affected auxin-regulated root developmental processes, root characters such as growth rate and gravitropism were measured and compared among wild type and three mutant lines.
Within 24 hours, the average elongation of wild type roots was about 8.86 mm. The average elongation of 32-2-4 roots was slightly less than wild type. However, the average elongation of 31-83-4-3 and 33-7-5(4) roots were about 5.74 mm and 1.50 mm respectively, both significantly less than wild type (Table 8).

To determine the gravitropism of wild type and three mutant lines, the 5 DAG seedlings were rotated 90° for horizontal angle measurements after 2, 4, 6 hours. Based on the angle measurement, there was no significant difference in gravitropic response between 32-2-4 and wild type. In contrast, the responses of 31-83-4-3 and 33-7-5(4) were smaller than wild type at all the time points and never reached the expected rotation of 90° (Table 8, Figure 7).

**DR5::GUS Expression is Reduced in 33-7-5(4)**

The DR5::GUS reporter gene enables relative levels of active auxin to be compared qualitatively (Ulmasov et al., 1999). To test if 33-7-5(4) leaves show altered auxin response, the homozygous population of 33-7-5(4) that expressed DR5::GUS were screened and a developmental series of roots and leaves of 33-7-5(4):DR5::GUS and wild type:DR5::GUS from 2 DAG to 5 DAG was compared (Figure 8). Within the 5 DAG root tips, the DR5::GUS expression was strongly reduced both in level (the intensity of color) and extent (the pattern of expression) in the mutant 33-7-5(4) compared to wild type (Figure 8 A, B). Within the 5 DAG wild type first leaves, DR5::GUS expression was intense and predicted the leaf vein pattern development (Figure 8 C); in contrast, no DR5::GUS expression was seen in 33-7-5-(4) (Figure 8 D), and the first leaf size was about the same size as 2 DAG to 3 DAG wild type first leaves (Figure 8 E, F).
To analyze auxin response level in 32-2-4, the mutants were crossed to DR5::GUS. In the F2 generation, 61 plants with 32-2-4 phenotypes were harvested and screened for homozygous GUS expression (expecting a quarter of 61). However, no homozygous plants were found which suggested that 32-2-4 was linked to DR5::GUS. The heterozygous seeds were saved for further screening but no further study of them was completed.

Double Mutants cuc2-3 32-2-4 has Smooth Leaf Margin

In order to determine if serrated leaf mutants can be affected by the leaf margin mutant cuc2-3 (smooth leaf margin with no serrations), crosses were made between cuc2-3 and mutant lines (cuc2-3×32-2-4 and cuc2-3×33-7-5(4)) to generate double mutants.

The wild type fifth leaves had an average of 4.76 serrations (n=17), the 32-2-4 homozygous fifth leaves had an average of 8.80 serrations (n=15) which was significantly more than wild type. The fifth leaves of double mutant cuc2-3 32-2-4 had no serrations like the single mutant cuc2-3 (Table 9, Figure 9). Thus, cuc2-3 was epistatic to the serration phenotype of 32-2-4.

Two hundred and sixteen (216) F2 seeds were planted to screen for the plants with 33-7-5(4) phenotype. Of these, fourteen (14) plants had the 33-7-5(4) phenotype. None of the F3 populations generated by these self-fertilizing plants segregated for cuc2-3. One possible explanation for this observation was that 33-7-5(4) was linked to cuc2-3. A second possible explanation was that 33-7-5(4) was epistatic to cuc2-3. Thus, no cuc2-3 33-7-5(4) double mutant was confirmed.

Mapping of 32-2-4 and 33-7-5(4)
Mutants 33-7-5(4) and 32-2-4 (Col ecotype) were crossed to the Landsberg *erecta* (Ler) ecotype wild type plants and the F2 plants were later planted as the mapping population. DNA was extracted from the leaves of F2 plants showing a clear mutant phenotype and used as PCR template (Dellaporta *et al.*, 1983). To determine the chromosomal location of the mutation, three to five primers on each chromosome were tested and used for amplifying the isolated mutant DNA (Table 1).

The genetic linkage was established by looking at the length of the PCR products on the agarose gel (Figure 10). For each pair of the SSLP markers, DNA samples from three controls (Col, Het (F1 of cross between wild type Col and Ler), Ler), and the mapping population were selected and amplified through the PCR reaction. Following gel electrophoresis of the PCR products, the genotypes of the mapping population were obtained by comparing the DNA band location of the mapping samples and controls. If the Col and Ler genotypic ratio was significantly different from 1:1 as determined by Pearson’s chi-squared test, I considered that this marker was linked with the mutant allele.

With a mapping population of 108 and 106 respectively, the 32-2-4 gene was mapped to chromosome II between markers 2-61 and 2-82, approximately 9.72 and 5.19 map units from markers 2-61 and 2-82 respectively. With a mapping population of 135, the 33-7-5(4) gene mapped to chromosome V between markers 5-85 and 5-116, approximately 16.30 and 9.63 map units from the markers 5-85 and 5-116 respectively (Table 10). From this mapping data, 33-7-5(4) is linked with *cuc2-3* (located on chromosome 5, AGI position 102.9 cM), thus explaining the lack of *cuc2-3* 33-7-5(4) double mutants.
Auxin is required for a number of key processes during plant development (Scarpella et al., 2010). Studies of a variety of Arabidopsis thaliana mutants indicate that defects in auxin biosynthesis, transport or response often result in aberrant plant development (Berná et al., 1999). I have identified four genetically stable mutants based on the first leaf phenotype. Mutant 115-21-4 had first leaves with frequent free-ending veins, and was determined to be an allele of FKDI by complementation testing. Mutant 31-83-4-3 had elongated first leaves with pointed leaf tips, frequent serrations and free-ending veins; mutant 32-2-4 had first leaves with small serrations and a simplified vein pattern; mutant 33-7-5(4) had small, pale first leaves with pointed leaf tips, frequent serrations, extremely simplified vein pattern and frequent free-ending secondary veins. These last three mutant lines (31-83-4-3, 32-2-4 and 33-7-5(4)) were further studied for shoot and root phenotypes. In addition, models to explain their first leaf development were proposed based on the phenotypic defects. Two mutant lines (32-2-4 and 33-7-5(4)) were mapped to the specific chromosomes using molecular markers.

31-83-4-3 Phenotype was Consistent with Defects in Auxin Transport

The first leaf of 31-83-4-3 had a pointed leaf tip and frequent serrations, and a simplified leaf vein pattern with frequent distal free ends in secondary veins. The first leaf phenotype suggested the 31-83-4-3 phenotype may have, like the unh and vam3-4 (vacuolar morphology) phenotype, resulted from expanded PIN1 within the leaf margin (Figure 2 F, H) (Shirakawa et al., 2009, Pahari et al., 2014). Alternatively (or additionally), the 31-83-4-3 phenotype may have resulted from defective auxin transport (lack of upward
auxin flux in the margin or in developing veins) possibly correlated with changes to PIN1 polarity; as compensation, auxin concentration maxima at the leaf marginal area form and induce serration outgrowth.

A series of shoot and root phenotypic defects in 31-83-4-3, such as increased numbers of rosette leaves, decreased internode elongation and increased branching (axillary bud growth) in the shoot, and reduced primary root growth and length, were consistent with an auxin transport defect. Auxin transport is required for cell division and elongation, which are correlated with the shoot and root elongation. Increased downward auxin transport reduces the auxin level in the shoot (resulting in a slower shoot growth rate) and increases the auxin level in root (inhibiting root growth) (Peer et al., 2011). The bushy appearance of mature 31-83-4-3 plants suggested that the lateral buds were released from the apical dominance; the increased lateral bud outgrowth either resulted from increased downward auxin transport (Prusinkiewicz et al., 2009) as suggested above for root growth or from the lack of upward auxin transport in the stem (Leyser, 2003). 31-83-4-3 developed more rosette leaves than wild-type and formed a bushy rosette with wavy leaf blades, these characters were also similar to the mutant short stem and midrib (ssm), an allele of VAM3/SYP22 which has been implicated in vesicle transport to the vacuole (Ohtomo et al., 2005).

The fact that 31-83-4-3 shared many phenotypic characters with both unh and vam3-4, suggested that 31-83-4-3 may, like unh and vam3 (Shirakawa et al., 2009, Pahari et al., 2014), result in higher levels of PIN1, thus increasing auxin flux through secondary veins and the shoot. A model of the first leaf of 31-83-4-3 with increased auxin level in the margin, as well as increased auxin flux through the secondary veins was proposed (Figure
11 A). I suggest that the increased auxin flux through veins might result in additional basal localized PIN1 and lack of apical localized PIN1, and increased serration and distal non-meeting veins as the consequences, similar to what happens in unh mutants (Pahari et al., 2014).

Other phenotypic defects such as delayed flowering time, reduced fertility and reduced gravitropic response, suggested the 31-83-4-3 mutant may also have defects in auxin response that were correlated with changes to auxin transport. The ARF family are auxin response factors and individual ARFs have unique and overlapping functions, and positively or negatively regulate gene expression. Delayed flowering and reduced fertility have been found in arf2 mutants (Ellis et al., 2005) which have auxin response defects, and these phenomena are also observed in 31-83-4-3. In addition, organ fusion was frequently observed in 31-83-4-3, which has been suggested to occur because of a defect in auxin regulated cell division (Peer et al., 2011). Crosses between 31-83-4-3 and Ler, as well as plants transgenic for PIN1:GFP or DR5::GUS were completed, however no seed was successfully generated due to the sterility of 31-83-4-3. My inability to generate a mapping population of 31-83-4-3 or integrate DR5::GUS into 31-83-4-3 limited further study of this mutant line. Additional back crosses may remove the sterility from 31-83-4-3.

**Serrated Leaf of 32-2-4 May Result from Higher Auxin in the Leaf Margin**

Relative to wild type, the 32-2-4 first leaf had serrations and a simplified vein pattern with closed vein loops. Since the mature plant phenotype of 32-2-4 was similar to wild type except for the serrated leaves and slightly pale color, and there was no significant difference in the gravity response of the root tip, I propose that there was no significant
defect in global auxin biosynthesis, transport or response levels in 32-2-4. The later rosette leaves of 32-2-4 had more serrations and the serrations were bigger than wild type; this phenotype suggested that 32-2-4 may have, like BODENLOS (BDL) mutants, increased auxin activity in the marginal area especially in the positions of the serrations (Bilsborough et al., 2011). I proposed a model of the 32-2-4 first leaf shape and vein pattern to show that the increased leaf serrations were correlated with increased marginal auxin maxima (Figure 11 B).

The CUC2 gene directs PIN1 re-localization within the leaf margin which determines the auxin transport direction, a process necessary for the formation of auxin convergence points at the leaf margin area where the serrations are forming (Bilsborough et al., 2011). A feedback loop among PIN1, auxin and CUC2 has been proposed to maintain a certain auxin concentration at the auxin convergence points for the serration growth (Bilsborough et al., 2011). The cuc2-3 mutant failed to develop serrations due to the lack of CUC2. The cuc2-3 phenotype was epistatic to 32-2-4 (Figure 9 D) which suggested the 32-2-4 was functioning in the same pathway as CUC2. The lack of serrations when 32-2-4 was combined with cuc2-3 suggested that relocalization of PIN1 by CUC2 was required for 32-2-4 to generate enlarged serrations, consistent with the idea that 32-2-4 increased auxin activity at auxin convergence points.

The delayed development of 32-2-4 (late flowering time which corresponds with small rosette leaves and increased leaf number) may be due to the pale color, and the pale color may result from lower chlorophyll content or lower cell density in the leaf blade. Thus, further study of mutant 32-2-4 should focus on the gene function in serration formation and the pale color. Mutant 32-2-4 mapped to the position between flanking
markers 2-61 and 2-82 on chromosome 2, approximately at 74.7cM on AGI Map. Based on the map position and the pale color phenotype, 32-2-4 could be an allele of some known genes such as ALBINO AND PALE GREEN10 (APG10) and LOWER CELL DENSITY1 (LCD1). The apg10 mutant has pale green cotyledons and rosette leaves, leaf development is delayed and the leaves are small, narrow, with some serrations (Noutoshi et al., 2005). Similar to 32-2-4, the lcd1 mutant plants are pale-green compared to wild type under optimal light intensity, and even paler under higher light intensities (Barth and Conklin, 2003).

**Auxin Response is Reduced in 33-7-5(4)**

33-7-5(4) had a flipper shaped first leaf, and the rosette leaves were small, pale and serrated. Both the shoot growth and root growth were slow, resulting in a weak plant with the developmental progress delayed about 4-5 days compared to wild type. At 5DAG, the DR5::GUS expression in the mutant 33-7-5(4) root tip was significantly reduced compared to the wild type root tip. This result suggested that the auxin response in 33-7-5(4) was lower than wild type which was consistent with the slower root gravitropic response. Thus, the gene 33-7-5(4) may not only function in leaf shape and vein patterning, but also in primary development or physiological processes which could alter overall auxin levels.

Due to the whole plant phenotype and the very low DR5::GUS expression in both roots and leaves, I suggest that overall low auxin level results in reduced cell division and expansion in 33-7-5(4) mutant. A possible explanation for the leaf phenotype is that, auxin levels in the leaf margin were above a threshold value that allowed serration outgrowth and the development of secondary veins, while auxin levels in the leaf blade were too low to
support sufficient cell division and/or expansion, as well as development of higher-order veins. Supporting the idea that 33-7-5(4) had reduced auxin response, its highly simplified vein pattern resembled mp mutants which produced fewer secondary veins and no veins along the leaf margin (Mattsson et al., 1999).

In contrast to the other two models I have proposed for the leaf serration formation, I propose that the serrations of 33-7-5(4) were due to the reduced growth of the leaf blade relative to the leaf margin. Thus, in the model of 33-7-5(4) first leaf shape and vein pattern formation, the auxin maxima that indicate the sites of serration on the margin were reduced relative to wild type and the other two mutants (31-83-4-3 and 32-2-4) (Figure 11 C).

Mutant 33-7-5(4) mapped to the position between flanking marker 5-85 and 5-116 on chromosome 5, approximately at 104.5 cM on AGI Map. Based on the mapping position of 33-7-5(4) and the identified position of CUC2 (102.9 cM on AGI Map), these two genes were linked which was consistent with my inability to obtain plants homozygous for cuc2-3 and 33-7-5(4).

Conclusions

In this study, four mutant lines were analyzed in detail: mutant 115-11-21-4 was an allele of FKDI with frequent distal free-ending veins, and the other three mutants were likely defective in auxin biosynthesis, transport or response. Mutant 31-83-4-3 likely had increased auxin flux through secondary veins and increased downward auxin transport through the shoot, possibly due to the increased PIN1 expression and/or altered PIN1 localization. Mutant 32-2-4 likely had no significant defect in global auxin biosynthesis, transport or response levels, but instead had increased auxin activity at marginal auxin
convergence points to enable the formation of increased leaf serrations. Mutant 33-7-5(4) may have had global reduction in auxin biosynthesis or response levels, thus the whole plant was weak and pale, with slower shoot and root growth rate and root gravitropism.
REFERENCES


<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>AGI BAC</th>
<th>Annealing Temp. (°C)</th>
<th>Mg²⁺ (mM)</th>
<th>Col¹ (bp)</th>
<th>Ler¹ (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-11 F</td>
<td>ACC CAA GTG ATC GCC ACC</td>
<td>F21M12</td>
<td>56.5</td>
<td>2.0</td>
<td>111</td>
<td>89</td>
</tr>
<tr>
<td>1-11 R</td>
<td>AAC CAA GGC ACA GAA GCG TGG GCA GCA TAG AGG CGA GGT TA</td>
<td>F7A19</td>
<td>54.2</td>
<td>2.5</td>
<td>210</td>
<td>169</td>
</tr>
<tr>
<td>1-15 F</td>
<td>GGT GGA TCG ACT GTG AGG TT TGG GGA AAT CAA AAC GCA GT</td>
<td>F20D23</td>
<td>53.8</td>
<td>2.5</td>
<td>156</td>
<td>141</td>
</tr>
<tr>
<td>1-15 R</td>
<td>TGC TTC AAT CAG GGA TTT CG TAG CCG AGA AGG GAT TGA TG</td>
<td>F28J9</td>
<td>55.0</td>
<td>2.5</td>
<td>255</td>
<td>211</td>
</tr>
<tr>
<td>1-52 F</td>
<td>TGG ATT CAA GAC CTA AAG CTG A</td>
<td>F11F12</td>
<td>55.5</td>
<td>2.5</td>
<td>327</td>
<td>273</td>
</tr>
<tr>
<td>1-52 R</td>
<td>TGG ATC ATC GAG GGA CTC AT TCA CGA TAC GTT CAA AAT TAG GG</td>
<td>F20P5</td>
<td>55.2</td>
<td>2.5</td>
<td>196</td>
<td>164</td>
</tr>
<tr>
<td>1-116 F</td>
<td>TGG ATG TAT TTT GCT AAT TGA GG</td>
<td>T8K22</td>
<td>50.6</td>
<td>2.5</td>
<td>213</td>
<td>217</td>
</tr>
<tr>
<td>1-116 R</td>
<td>CCT TCA CAT CCA AAA CCC AC CCC AAA AGT TAA TTA TAC TGT</td>
<td>T18C20</td>
<td>51.8</td>
<td>2.5</td>
<td>105</td>
<td>90</td>
</tr>
<tr>
<td>2-11 R</td>
<td>CCT CCA AAC CAG CTA TCG TCG GAG ATA CCC GAG CTA AA</td>
<td>F23M2</td>
<td>53.2</td>
<td>2.5</td>
<td>384</td>
<td>316</td>
</tr>
<tr>
<td>2-23 F</td>
<td>CAA AAG CCA CAC TAA GGG ACA</td>
<td>F3K23</td>
<td>52.0</td>
<td>2.5</td>
<td>396</td>
<td>328</td>
</tr>
<tr>
<td>2-23 R</td>
<td>TGC ACC ATT GAG ACA AGC TC GCA CAG TCC AAG TCA CAA CC</td>
<td>F3C11</td>
<td>53.4</td>
<td>2.0</td>
<td>189</td>
<td>213</td>
</tr>
<tr>
<td>2-50 F</td>
<td>CGC TAC GCT TTT CGG TAA AG GTT CAT GGA CGG ATG TGT ATG C</td>
<td>T6B20</td>
<td>55.4</td>
<td>2.5</td>
<td>75</td>
<td>83</td>
</tr>
<tr>
<td>2-50 R</td>
<td>CTA GTG GTG GTT AAA ATA TAC GC CTC ATC TTG TTT ACT AAT ATC AAC TCG TTT AAT CAT TGT TTT GTG CGA AAA T</td>
<td>F6E13</td>
<td>51.0</td>
<td>2.5</td>
<td>196</td>
<td>168</td>
</tr>
<tr>
<td>2-3 R⁴</td>
<td>TTT GAA CTG CAA TTG ATG GA TAT AGG GCA AGC CCA AGA TG</td>
<td>T2O4</td>
<td>48.3</td>
<td>2.5</td>
<td>340</td>
<td>244</td>
</tr>
<tr>
<td>3-42 F</td>
<td>CCC CGA GTT GAG GTA TT GAA GAA ATT CCT AAA GCA TTC</td>
<td>MFE16</td>
<td>56.5</td>
<td>2.5</td>
<td>180</td>
<td>230</td>
</tr>
<tr>
<td>3-42 R</td>
<td>GAA TCA GTC ACC TTT GT GCT GCA ATG CCA TCT TGA TA</td>
<td>MSJ3</td>
<td>54.2</td>
<td>2.5</td>
<td>156</td>
<td>176</td>
</tr>
<tr>
<td>3-68.5 F⁴</td>
<td>GTC GAT TGA TGT TTT GGA CC</td>
<td>T10D17</td>
<td>53.8</td>
<td>2.5</td>
<td>850</td>
<td>1000</td>
</tr>
</tbody>
</table>

Table 1. SSLP markers designed and used in mapping 32-2-4 and 33-7-5(4)
<table>
<thead>
<tr>
<th>AGI</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing Temp</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Length 1</th>
<th>Length 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-68.5 R</td>
<td>CCC TAC ATT CTA CAA CCA</td>
<td>TGT AGC C</td>
<td>T6H20</td>
<td>55.0</td>
<td>2.5</td>
<td>370</td>
</tr>
<tr>
<td>3-74 F</td>
<td>TGC ATT GGT TTC TCT GCT TG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-74 R</td>
<td>TGC ACA CTC ATG GTT TCC TC</td>
<td>ATG GAG AAG CTT ACA CTC</td>
<td>T17J13</td>
<td>55.5</td>
<td>2.5</td>
<td>143</td>
</tr>
<tr>
<td>3-86 F</td>
<td>TGG ATT TCT TCC TCT CTT CAC</td>
<td>ATC</td>
<td>T20010</td>
<td>55.2</td>
<td>2.5</td>
<td>235</td>
</tr>
<tr>
<td>3-88 F</td>
<td>TCT TCC AAA CCA TGC AAT GA</td>
<td>TGG CAG GGG AAT AGA TGA</td>
<td>T7B11</td>
<td>50.6</td>
<td>2.5</td>
<td>274</td>
</tr>
<tr>
<td>4-11 F</td>
<td>GAC AAT AAC CTT GCG TGG</td>
<td>TAC A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-11 R</td>
<td>TGG TTA CTG TAT GCC AAA TGA A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-31 F</td>
<td>ACA TTT GGT GGG CGA GTA AC</td>
<td>TAG AGG GAA CAA TCG GAT GC</td>
<td>F24G24</td>
<td>51.8</td>
<td>2.5</td>
<td>333</td>
</tr>
<tr>
<td>4-31 R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-60 F</td>
<td>ACT TGC TTT CGC TTT GCA GT</td>
<td>TGG CCT TTT GCT CTC TGT TT</td>
<td>F27B13</td>
<td>53.2</td>
<td>2.5</td>
<td>353</td>
</tr>
<tr>
<td>5-13 F</td>
<td>TTT CTC TGT TGG GGC AAA AC</td>
<td>AGG CAG CCG CAT CTT TAA TA</td>
<td>MBK20</td>
<td>52.0</td>
<td>2.5</td>
<td>384</td>
</tr>
<tr>
<td>5-13 R</td>
<td>TGG ATC TCG GCT ATT GAT TGC</td>
<td>TCC CAA ATC AAT TCA AGG AAA</td>
<td>F2P16</td>
<td>53.0</td>
<td>2.5</td>
<td>345</td>
</tr>
<tr>
<td>5-51 F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-51 R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-85 F</td>
<td>GAT TTC GCT CTC TGC CAA AA</td>
<td>ACA AAA TAC AAG CCC AAC AAG T</td>
<td>K23L20</td>
<td>55.4</td>
<td>2.5</td>
<td>345</td>
</tr>
<tr>
<td>5-85 R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-115 F</td>
<td>CCA CAT TTT CTT CTT CTT ATA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-115 R</td>
<td>CAA CAT TTA GCA AAT CAA CTT</td>
<td>MSL3</td>
<td>51.0</td>
<td>2.0</td>
<td>137</td>
<td>133</td>
</tr>
<tr>
<td>5-116 F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-116 R</td>
<td>GAA CAC ATG GAA TCT TCA CAT CA</td>
<td></td>
<td>MAC9</td>
<td>48.3</td>
<td>2.5</td>
<td>300</td>
</tr>
</tbody>
</table>

The AGI BAC indicates the bacterial artificial chromosome where the primer pair is located on the *Arabidopsis* Genome Initiative map. The annealing temperature and Mg<sup>2+</sup> concentration indicate the variations from the standard PCR reaction.

* Indicates the length of final DNA fragment of each ecotype.

a New markers designed for this study.
Table 2. Segregation analysis of various mutant families

<table>
<thead>
<tr>
<th>F2 Population</th>
<th>WT Count</th>
<th>Mutant Count</th>
<th>Approximate Ratio (WT: Mutant)</th>
<th>Pearson’s Chi-squared Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>31-83-4-3 bc3F2</td>
<td>178</td>
<td>20</td>
<td>8.90 : 1</td>
<td>23.44, p &lt; 0.001*</td>
</tr>
<tr>
<td>32-2-4 bc4F2</td>
<td>153</td>
<td>54</td>
<td>2.83 : 1</td>
<td>0.13, p = 0.718</td>
</tr>
<tr>
<td>33-7-5(4) bc4F2</td>
<td>149</td>
<td>21</td>
<td>7.10 : 1</td>
<td>14.50, p &lt; 0.001*</td>
</tr>
<tr>
<td>115-11-21-4 bc2F2</td>
<td>183</td>
<td>65</td>
<td>2.82 : 1</td>
<td>0.19, p = 0.660</td>
</tr>
</tbody>
</table>

All the plants were scored at 21 DAG, except 33-7-5(4), which were 28 DAG due to the slower growth. Pearson’s chi-squared test (one-tailed) was applied based on the expected 3: 1 ratio. * Indicates this value is significantly different from the expectation (p < 0.05).
Table 3. Complementation data of *115-11-21-4*

<table>
<thead>
<tr>
<th>Cross (Female × Male)</th>
<th>F1 Progeny Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>115-11-21-4×fkd1</em></td>
<td>Mutant (62)</td>
</tr>
<tr>
<td><em>115-11-21-4×sfc</em></td>
<td>Wild Type (51)</td>
</tr>
</tbody>
</table>

Number in bracket represents number of plants scored.
Table 4. First leaf shape characters of various genotypes at 21 DAG<sup>a</sup>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild Type (30)</th>
<th>31-83-4-3 (30)</th>
<th>32-2-4 (30)</th>
<th>33-7-5(4) (30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serrations</td>
<td>0</td>
<td>1.60±0.16</td>
<td>1.70±0.15</td>
<td>1.87±0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.001&lt;sup&gt;$&lt;/sup&gt;</td>
<td>p &lt; 0.001&lt;sup&gt;$&lt;/sup&gt;</td>
<td>p &lt; 0.001&lt;sup&gt;$&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hydathodes</td>
<td>3.00±0.07</td>
<td>1.63±0.14</td>
<td>3.27±0.08</td>
<td>2.60±0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.001&lt;sup&gt;*&lt;/sup&gt;</td>
<td>p = 0.015&lt;sup&gt;*&lt;/sup&gt;</td>
<td>p = 0.014&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Width (W, mm)</td>
<td>6.55±0.07</td>
<td>4.19±0.16</td>
<td>4.94±0.06</td>
<td>3.06±0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.001&lt;sup&gt;*&lt;/sup&gt;</td>
<td>p &lt; 0.001&lt;sup&gt;*&lt;/sup&gt;</td>
<td>p &lt; 0.001&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Length (L, mm)</td>
<td>7.21±0.10</td>
<td>6.24±0.18</td>
<td>5.94±0.07</td>
<td>3.93±0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.001&lt;sup&gt;*&lt;/sup&gt;</td>
<td>p &lt; 0.001&lt;sup&gt;*&lt;/sup&gt;</td>
<td>p &lt; 0.001&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>L/W</td>
<td>1.10±0.01</td>
<td>1.52±0.04</td>
<td>1.20±0.01</td>
<td>1.31±0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.001&lt;sup&gt;*&lt;/sup&gt;</td>
<td>p &lt; 0.001&lt;sup&gt;*&lt;/sup&gt;</td>
<td>p &lt; 0.001&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values present means ± s.e.m.
Number in bracket represents number of plants scored.
<sup>a</sup> 33-7-5(4) were scored at 28 DAG due to the slower growth.
<sup>$</sup> Indicates significant difference from wild type based on two-tailed Fisher’s exact test.
<sup>*</sup> Indicates significant difference from wild type based on two-tailed Student’s t-test.
Significance level: p < 0.05.
Table 5. First leaf vascular pattern characters of various genotypes at 21 DAG\textsuperscript{a}

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild Type (30)</th>
<th>31-83-4-3 (30)</th>
<th>32-2-4 (30)</th>
<th>33-7-5(4) (30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Areoles</td>
<td>21.03±0.72</td>
<td>9.27±0.76, p &lt; 0.001*</td>
<td>14.47±0.51, p &lt; 0.001*</td>
<td>4.63±0.48, p &lt; 0.001*</td>
</tr>
<tr>
<td>Secondary Veins</td>
<td>9.03±0.27</td>
<td>6.93±0.26, p &lt; 0.001*</td>
<td>8.17±0.18, p = 0.009*</td>
<td>3.93±0.16, p &lt; 0.001*</td>
</tr>
<tr>
<td>Tertiary Veins</td>
<td>25.20±0.87</td>
<td>10.10±0.86, p &lt; 0.001*</td>
<td>17.53±0.46, p &lt; 0.001*</td>
<td>6.03±0.56, p &lt; 0.001*</td>
</tr>
<tr>
<td>Quaternary Veins</td>
<td>7.00±0.50</td>
<td>1.73±0.40, p &lt; 0.001*</td>
<td>4.57±0.35, p &lt; 0.001*</td>
<td>1.07±0.24, p &lt; 0.001*</td>
</tr>
<tr>
<td>Free Ends</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondaries (%)</td>
<td>1.10±0.19 (12.18%)</td>
<td>1.93±0.24 (27.88%)</td>
<td>1.13±0.15 (13.50%)</td>
<td>0.77±0.14 (19.49%)</td>
</tr>
<tr>
<td></td>
<td>p = 0.008*</td>
<td>p = 0.892</td>
<td>p = 0.170</td>
<td></td>
</tr>
<tr>
<td>Tertiaries (%)</td>
<td>12.73±0.60 (50.53%)</td>
<td>5.87±0.53 (58.09%)</td>
<td>10.40±0.48 (59.17%)</td>
<td>4.70±0.35 (77.90%)</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001*</td>
<td>p = 0.004*</td>
<td>p &lt; 0.001*</td>
<td></td>
</tr>
<tr>
<td>Quaternaries (%)</td>
<td>6.43±0.43 (91.90%)</td>
<td>1.57±0.35 (90.38%)</td>
<td>4.23±0.30 (92.48%)</td>
<td>0.93±0.19 (87.50%)</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001*</td>
<td>p &lt; 0.001*</td>
<td>p &lt; 0.001*</td>
<td></td>
</tr>
<tr>
<td>Midvein Extension</td>
<td>0</td>
<td>0.40±0.09, p &lt; 0.001\§</td>
<td>0</td>
<td>0.40±0.09, p &lt; 0.001\§</td>
</tr>
<tr>
<td>Branch Points</td>
<td>62.33±1.83</td>
<td>28.30±1.93, p &lt; 0.001*</td>
<td>44.70±0.88, p &lt; 0.001*</td>
<td>16.07±1.24, p &lt; 0.001*</td>
</tr>
<tr>
<td>Vascular Islands</td>
<td>0</td>
<td>0.37±0.12, p = 0.005\§</td>
<td>0.17±0.07, p = 0.052</td>
<td>0</td>
</tr>
<tr>
<td>Marginal Venation Gaps</td>
<td>0</td>
<td>0.97±0.24, p &lt; 0.001\§</td>
<td>0</td>
<td>1.17±0.18, p &lt; 0.001\§</td>
</tr>
</tbody>
</table>

Values present means ± s.e.m.
The number in brackets beside the genotype represents the number of plants scored.
The number in brackets beside the free ends represents the percentage of free-ending veins.
\textsuperscript{a} 33-7-5(4) were scored at 28 DAG due to the slower growth.
* Indicates significant difference from wild type based on two-tailed Student’s t-test.
\§ Indicates significant difference from wild type based on two-tailed Fisher’s exact test.
Significance level: p < 0.05.
Table 6. Cotyledon vascular pattern characters of various genotypes at 14 DAG\(^a\)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild Type (30)</th>
<th>31-83-4-3 (30)</th>
<th>32-2-4 (30)</th>
<th>33-7-5(4) (30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Areoles</td>
<td>3.07±0.12</td>
<td>2.10±0.13, (p &lt; 0.001^*)</td>
<td>3.00±0.13, (p = 0.700)</td>
<td>0.53±0.16, (p &lt; 0.001^*)</td>
</tr>
<tr>
<td>Secondary Veins</td>
<td>3.13±0.11</td>
<td>3.27±0.16, (p = 0.499)</td>
<td>2.97±0.14, (p = 0.360)</td>
<td>2.13±0.18, (p &lt; 0.001^*)</td>
</tr>
<tr>
<td>Tertiary Veins</td>
<td>1.13±0.16</td>
<td>0.87±0.15, (p = 0.235)</td>
<td>1.3±0.17, (p = 0.489)</td>
<td>0.03±0.03, (p &lt; 0.001^*)</td>
</tr>
<tr>
<td>Quaternary Veins</td>
<td>0.10±0.06</td>
<td>0, (p = 0.237)</td>
<td>0, (p = 0.237)</td>
<td>0, (p = 0.237)</td>
</tr>
<tr>
<td>Free Ends</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distal Secondaries (%)</td>
<td>0.07±0.07 (2.13%)</td>
<td>0.97±0.16 (29.59%), (p &lt; 0.001^*)</td>
<td>0.10±0.06 (4.44%)</td>
<td>1.37±0.16 (64.06%), (p &lt; 0.001^*)</td>
</tr>
<tr>
<td>Proximal Secondaries (%)</td>
<td>0</td>
<td>0.23±0.10 (7.14%), (p = 0.052)</td>
<td>0</td>
<td>0.23±0.09 (10.94%), (p = 0.024^S)</td>
</tr>
<tr>
<td>Tertiaries (%)</td>
<td>1.10±0.17 (97.06%)</td>
<td>0.83±0.14 (96.15%), (p = 0.234)</td>
<td>1.13±0.17 (85.71%)</td>
<td>0.03±0.03 (100%), (p &lt; 0.001^*)</td>
</tr>
<tr>
<td>Quaternaries (%)</td>
<td>0.10±0.06 (100%)</td>
<td>0, (p = 0.237)</td>
<td>0, (p = 0.237)</td>
<td>0, (p = 0.237)</td>
</tr>
<tr>
<td>Midvein Extension</td>
<td>0.07±0.05</td>
<td>0.80±0.07, (p &lt; 0.001^*)</td>
<td>0.07±0.05, (p = 1)</td>
<td>0.20±0.07, (p = 0.134)</td>
</tr>
<tr>
<td>Branch Points</td>
<td>7.47±0.17</td>
<td>7.03±0.29, (p = 0.204)</td>
<td>7.30±0.18, (p = 0.505)</td>
<td>2.90±0.32, (p &lt; 0.001^*)</td>
</tr>
</tbody>
</table>

Values present means ± s.e.m. For the row of Genotype, number in bracket represents the number of plants scored.
For the rows of Free Ends, the number in bracket represents the percentage of free-ending veins.
\(^a\) 33-7-5(4) were scored at 21 DAG due to the slower growth.
\(^*\) Indicates significant difference from wild type based on two-tailed Student’s \(t\)-test.
\(^S\) Indicates significant difference from wild type based on two-tailed Fisher’s exact test.
Significance level: \(p < 0.05\).
Table 7. Adult plant shoot characters of various genotypes at 35 DAG

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild Type (36)</th>
<th>31-83-4-3 (37)</th>
<th>32-2-4 (39)</th>
<th>33-7-5(4) (37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flowering Time (DAG)</td>
<td>20.94±0.31</td>
<td>28.68±0.31</td>
<td>22.56±0.42</td>
<td>30.30±0.41</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p = 0.003</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Stem Branches</td>
<td>3.44±0.09</td>
<td>7.27±0.35</td>
<td>3.15±0.11</td>
<td>4.27±0.17</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p = 0.053</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Rosette Leaves</td>
<td>6.56±0.18</td>
<td>8.97±0.24</td>
<td>7.79±0.27</td>
<td>8.08±0.24</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Fused Leaf and Stem</td>
<td>0</td>
<td>0.54±0.10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p = 1</td>
<td>p = 1</td>
<td>p = 1</td>
</tr>
</tbody>
</table>

Values present means ± s.e.m. Number in bracket represents number of plants scored. a Excludes flowering time. * Indicates significant difference from wild type based on two-tailed Student’s t-test. $ Indicates significant difference from wild type based on two-tailed Fisher’s exact test. Significance level: p < 0.05.
Table 8. Root characters of various genotypes from 4 DAG to 5 DAG

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild Type (30)</th>
<th>31-83-4-3 (25)</th>
<th>32-2-4 (30)</th>
<th>33-7-5(4) (30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root Elongation within 24 hours (from 4 DAG to 5 DAG, mm)</td>
<td>8.86±0.29</td>
<td>5.74±0.37</td>
<td>8.14±0.21</td>
<td>1.50±0.16</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001*</td>
<td>p = 0.050*</td>
<td>p &lt; 0.001*</td>
<td></td>
</tr>
<tr>
<td>Gravitropism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horizontal Angle (°) of Root Tip after Rotated 90°</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2hr</td>
<td>46.26±1.85</td>
<td>16.91±3.87</td>
<td>44.78±2.72</td>
<td>5.08±2.12</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001*</td>
<td>p = 0.655</td>
<td>p &lt; 0.001*</td>
<td></td>
</tr>
<tr>
<td>4hr</td>
<td>74.40±1.58</td>
<td>43.50±4.36</td>
<td>75.49±2.62</td>
<td>15.67±2.67</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001*</td>
<td>p = 0.725</td>
<td>p &lt; 0.001*</td>
<td></td>
</tr>
<tr>
<td>6hr</td>
<td>86.00±1.42</td>
<td>60.31±4.05</td>
<td>86.92±2.18</td>
<td>25.43±3.52</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001*</td>
<td>p = 0.726</td>
<td>p &lt; 0.001*</td>
<td></td>
</tr>
</tbody>
</table>

Values present means ± s.e.m, number in bracket represents number of plants scored.

*a Roots were rotated 90° at 5 DAG and captured for gravitropism measurements after 2, 4, 6 hours.

*b Indicates significant difference from wild type based on two-tailed Student’s t-test (p < 0.05).
Table 9. Fifth leaf serrations numbers of various genotypes at 35 DAG

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild Type (17)</th>
<th>cuc2-3 (15)</th>
<th>32-2-4 (15)</th>
<th>32-2-4×cuc2-3 (15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serrations</td>
<td>4.76±0.34</td>
<td>0</td>
<td>8.80±0.30</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001$</td>
<td>p &lt; 0.001*</td>
<td>p &lt; 0.001$</td>
<td></td>
</tr>
</tbody>
</table>

Values present means ± s.e.m.
Number in bracket represents number of plants scored.
$ Indicates significant difference from wild type based on two-tailed Fisher’s exact test.
* Indicates significant difference from wild type based on two-tailed Student’s t-test.
Significance level: p < 0.05.
Table 10. Mapping distances between the mutations and the primer pairs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>32-2-4</th>
<th>33-7-5(4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer Pair</td>
<td>2-61 F, 2-61 R</td>
<td>5-85 F, 5-85 R</td>
</tr>
<tr>
<td></td>
<td>2-82 F, 2-82 R</td>
<td>5-115 F, 5-115 R</td>
</tr>
<tr>
<td>Number of Plants Scored</td>
<td>108</td>
<td>135</td>
</tr>
<tr>
<td>Col : Ler Ratio</td>
<td>195:21</td>
<td>226:44</td>
</tr>
<tr>
<td></td>
<td>201:11</td>
<td>141:11</td>
</tr>
<tr>
<td>Pearson’s chi-squared Test</td>
<td>140.17 *</td>
<td>122.68 *</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001*</td>
<td>p &lt; 0.001*</td>
</tr>
<tr>
<td>Map Distance (cM)</td>
<td>9.72</td>
<td>16.30</td>
</tr>
<tr>
<td></td>
<td>5.19</td>
<td>7.24</td>
</tr>
</tbody>
</table>

Pearson’s chi-squared test (one-tailed) was applied based on the expected 1: 1 ratio.
* Indicates this value is significantly different from the expectation (p < 0.05).
Figure 1. Diagrams of different leaf shape and leaf characters.
(A) Simple leaf with smooth margin, (B) Simple leaf with serrations, (C) Simple leaf with lobes, (D) Compound leaf, (E) *Arabidopsis thaliana* first leaf that shows the various vein pattern and leaf shape characters: the left half is presenting the wild type; the right half is presenting the potential mutant.
Figure 2. Models of leaf shape and vein pattern formation. 
(A) Leaf initials at the flanks of SAM (purple) correspond to the site of elevated auxin activity (green), the white arrow indicates the auxin transport direction resulting from PIN1 polarity (black arrows). (B) Early leaf primordium (grey): a maximum of auxin activity forms at the tip of the primordium; and auxin is drained through the centre of the primordium, marking the position of midvein (white arrows). (C) Primary morphogenesis: lateral vein (blue) formation and positions of serration development are correlated with auxin activity maxima at the margin. (D) The feedback loop among PIN1, auxin and CUC2: the polar (apical or basal) localization of PIN1 (yellow) determines auxin transport (red arrows), auxin flow enhances PIN1 localization with the same polarity (black arrows); CUC2 enables reorientation of PIN1 (dashed black arrows) and, in turn, auxin inhibits CUC2, thus stabilizing the position of auxin maxima (dark green)/CUC2 minima (pink) (predicting serrations) and auxin minima (bright green)/CUC2 maxima (magenta) (predicting sinuses). (E) In wild type first leaf, the apical maximum and lateral maxima (green) initiate the formation of midvein (dark grey) and first pair of secondary veins (blue). (F) In unh first leaf, the lateral maxima (green) is stronger than wild type. (G) At a later stage of wild type first leaf, the switch of PIN1 polarity leads the auxin transport direction toward to the distal part of midvein and gives rise to closed vein loop (purple). (H) At a later stage of unh first leaf, the expanded PIN1 expression (green) in the margin results stronger downward auxin flux and prevents the upward auxin flux, results the serration formation and free-ending secondary veins (Scarpella et al., 2010, Bilsborough et al., 2011, Pahari et al., 2014).
Figure 3. Complementation test of mutant 115-11-21-4 to fkd1 and sfc. (A) 115-11-21-4 has elongated first leaf shape and frequently exhibits distal free ends of secondary and tertiary veins, (B) fkd1 first leaf, (C) sfc first leaf, (D) wild type first leaf, (E) F1 generation from crossing of 115-11-21-4 and fkd1 was mutant phenotype with frequent free ending veins in first leaf, thus 115-11-21-4 was an allele of fkd1, (F) F1 generation from crossing of 115-11-21-4 and sfc was wild type phenotype, thus 115-11-21-4 was not an allele of sfc. Scale bars = 1 mm.
Figure 4. Cleared first leaves of various genotypes viewed under dissecting light microscope.
(A) wild type, (B,C) 31-83-4-3, (D) 32-2-4, (E,F) 33-7-5(4). All first leaves were removed at 21 DAG, except 33-7-5(4), which were removed at 28 DAG. Black arrows indicate the midvein extension and vascular island. Scale bars = 1 mm.
Figure 5. Cleared cotyledons of various genotypes viewed under dissecting light microscope.

(A-D) wild type, (E-H) 31-83-4-3, red arrows indicate the midvein extension, dashed yellow lines marked the normal midvein location, (I-L) 32-2-4, red arrows indicate the areoles formed by tertiary veins, (M-P) 33-7-5(4). All cotyledons were removed at 14 DAG, except 33-7-5(4), which were removed at 21 DAG. Scale bars = 1 mm.
Figure 6. Shoot phenotype of various genotypes at 35 DAG. (A) wild type, (B) 31-83-4-3, white square magnified the fused leaf and stem, (C) 32-2-4, (D) 33-7-5(4). Plants were grown in soil. Scale bars = 1 cm.
Figure 7. Root tip bend after being rotated 90° for 2, 4, 6 hours of various genotypes: wild type, 31-83-4-3, 32-2-4 and 33-7-5(4) as viewed under dissecting light microscope. The red dots in each picture indicated the root tip position at 4 DAG. All the roots were rotated at 5 DAG. Scale bar = 5 mm.
Figure 8. DR5::GUS expression in 33-7-5(4) and wild type root tips and first leaves. At 5 DAG, DR5::GUS expression in (A) 33-7-5(4) root tip is reduced compared to (B) wild type. (C) DR5::GUS expression in the 5 DAG first leaves of wild type is presented and predicts the position of leaf vein formation. (D) No DR5::GUS expression is seen in 33-7-5-(4) 5 DAG first leaves, and the first leaf size is about the same size as 2 DAG (E) to 3 DAG (F) wild type first leaves. Scale bar = 100 μm.
Figure 9. Fifth leaf phenotypes of various genotypes at 35 DAG. (A) wild type, (B) cuc2-3, (C) 32-2-4, more serration on the distal part of the leaf blade as compared to wild type (D) double mutant cuc2-3 32-2-4. The serrations of 32-2-4 are suppressed by cuc2-3. Scale bar = 5 mm.
Figure 10. PCR products defining molecular markers used in mapping. SSLP marker linked to tested mutant showing the banding pattern of controls (Col, Het (Col×Ler F1), and Ler) and mutant×Ler F2 mapping samples.
Figure 11. Models for first leaf shape and vein pattern formation associated with defective auxin regulation in three mutant lines. (A) The serrations of 31-83-4-3 first leaf may result from higher PIN1 expression in the leaf margin, thus the increased auxin maxima on the leaf margin (green) induces the serration outgrowth; and the increased the auxin flux through secondary veins prevents the upward auxin transport (pink with a red cross mark), result the frequent distal free-ending veins. (B) No defects were determined in overall auxin level of 32-2-4, the serrated leaf shape results from increased auxin activity at the auxin convergence points in the leaf margin. (C) Extremely reduced auxin level in 33-7-5(4) limits the growth of leaf blade, and the auxin maxima on the leaf margin (light green) is reduced relative to wild type and other two mutants (31-83-4-3 and 32-2-4). Thus the serration may not result from the outgrowth of the margin, instead it may result from slow cell division and expansion in the central lamina.
APPENDIX 1. Selected mutant lines with leaf shape and/or vein pattern defects

<table>
<thead>
<tr>
<th>Mutant Lines</th>
<th>Previous Generation</th>
<th>Current Generation</th>
<th>Complementation Test</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>31-83-4-3</td>
<td>bc1F3</td>
<td>bc4F2</td>
<td>Not an allele of <em>fkd1, unh, gsy</em></td>
<td>Serrated first leaf with non-meeting secondary veins</td>
</tr>
<tr>
<td>32-1-4(1)</td>
<td>bc2F2</td>
<td>bc2F3</td>
<td>Allele of <em>gsy</em></td>
<td>Seeds from mutants were harvested and saved as bc2F3</td>
</tr>
<tr>
<td>32-2-4</td>
<td>bc3F4</td>
<td>bc5F2</td>
<td><strong>Not completed</strong></td>
<td><strong>Serrated first leaf, simplified vain pattern</strong></td>
</tr>
<tr>
<td>32-26-1(3)</td>
<td>bc1F2</td>
<td>bc2F3</td>
<td>Not an allele of <em>fkd1, unh, gsy</em></td>
<td>Small round thick purple leaf; nearly only one stem; short siliques</td>
</tr>
<tr>
<td>32-28</td>
<td>bc2F2</td>
<td>bc2F3</td>
<td><strong>Not completed</strong></td>
<td>No phenotype in bc2F2, M4 seeds did not germinate</td>
</tr>
<tr>
<td>32-28-1</td>
<td>bc1F2</td>
<td>bc2F3</td>
<td>Not an allele of <em>fkd1, unh, gsy</em></td>
<td>No abnormal phenotype in bc2F2</td>
</tr>
<tr>
<td>33-7-5(4)</td>
<td>bc3F2</td>
<td>bc5F2</td>
<td><strong>Not completed</strong></td>
<td><strong>Serrated first leaf with non-meeting secondary veins</strong></td>
</tr>
<tr>
<td>33-17-9(2)</td>
<td>bc1F2</td>
<td>bc2F3</td>
<td><strong>Not completed</strong></td>
<td>No abnormal phenotype in bc2F2</td>
</tr>
<tr>
<td>115-11-21-4</td>
<td>M4</td>
<td>bc2F3</td>
<td><strong>Allele of fkd1</strong></td>
<td><strong>Frequent distal free-ending veins</strong></td>
</tr>
<tr>
<td>226(7)</td>
<td>M5</td>
<td>bc1F2</td>
<td><strong>Not completed</strong></td>
<td>Small rosette leaves, floristic defects</td>
</tr>
<tr>
<td>es30</td>
<td>bc2F1</td>
<td>bc4F3</td>
<td>Not an allele of <em>fkd1, unh, gsy</em></td>
<td>Simple vein pattern, curved later rosette leaves, bushy</td>
</tr>
<tr>
<td>es31</td>
<td>M3</td>
<td>bc2F3</td>
<td>Not an allele of <em>fkd1, unh, gsy</em></td>
<td>No abnormal phenotype in bc2F2</td>
</tr>
<tr>
<td>es32</td>
<td>M4</td>
<td>bc1F3</td>
<td>Not an allele of <em>fkd1, unh, gsy</em></td>
<td>No abnormal phenotype in bc2F3</td>
</tr>
<tr>
<td>es33</td>
<td>bc1F2</td>
<td>bc2F3</td>
<td>Not an allele of <em>fkd1, unh, gsy</em></td>
<td>(Looks like bc1F3)gl1:12,14, No.1 crossed to everything</td>
</tr>
<tr>
<td>es33-1</td>
<td>bc1F2</td>
<td>bc2F3</td>
<td>Not an allele of <em>fkd1, unh, gsy</em></td>
<td>No phenotype in bc2F2</td>
</tr>
<tr>
<td>es39-4</td>
<td>M3</td>
<td>bc2F3</td>
<td>Not an allele of <em>fkd1, unh, gsy</em></td>
<td>Parallel simple veins, late flowering</td>
</tr>
<tr>
<td>es39-4(9)</td>
<td>bc1F1</td>
<td>bc2F3</td>
<td>Not an allele of <em>unh</em></td>
<td>Serrated leaf, simple vein, no abnormal phenotype in bc2F2</td>
</tr>
<tr>
<td>es39-6</td>
<td>M3</td>
<td>bc2F3</td>
<td>Not an allele of <em>fkd1, unh, gsy</em></td>
<td>Needle shape first leaf/no first leaf, serrated later leaves, weak</td>
</tr>
<tr>
<td>es39-6(8)</td>
<td>bc1F1</td>
<td>bc3F2</td>
<td>Not an allele of <em>fkd1, unh, gsy</em></td>
<td>F2 seeds collected, need to select phenotype and cross</td>
</tr>
<tr>
<td>es47-9</td>
<td>M4</td>
<td>bc1F3</td>
<td><strong>Not completed</strong></td>
<td>No abnormal phenotype in bc1F2</td>
</tr>
<tr>
<td>es48-15</td>
<td>M4</td>
<td>bc2F3</td>
<td>Not an allele of <em>fkd1, unh, gsy</em></td>
<td>No.8 hydathodes</td>
</tr>
<tr>
<td>es51-20</td>
<td>bc1F2</td>
<td>bc2F3</td>
<td>Not an allele of <em>fkd1</em></td>
<td>Simple vein pattern, round leaf shape, no-meeting distal vein</td>
</tr>
<tr>
<td>es74</td>
<td>M3</td>
<td>bc1F3</td>
<td><strong>Not completed</strong></td>
<td>No abnormal phenotype in bc1F2</td>
</tr>
<tr>
<td>es74-16</td>
<td>bc1F2</td>
<td>bc1F4</td>
<td><strong>Not completed</strong></td>
<td>No abnormal phenotype in leaves, but something in stem</td>
</tr>
</tbody>
</table>